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**UPTAKE AND UTILISATION OF
AMINO ACIDS
BY HUMAN HAIR FOLLICLES
AND RELATED CELLS**

CAROLINE RICHES B.A.

Thesis submitted for the degree of
Master of Philosophy

Open University
in collaboration with
Oxford University Laboratory of Physiology

January 1996

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ABSTRACT

Uptake and Utilisation of Amino Acids by Human Hair Follicles and Related Cells

The requirements of amino acids as components of hair proteins, and the utilisation of glutamine by hair follicles to provide energy, point to the importance of amino acids in hair follicle metabolism. This thesis describes an investigation of the role of amino acids in hair follicles, with particular regard to cysteine and arginine. The cellular transport of amino acids into the outer root sheath (ORS) is examined, since this regulates their intracellular availability, and may thus affect hair formation.

The major findings of this study are as follows:

Both arginine and cysteine are essential for hair growth and protein synthesis. Cysteine plays a role in determining hair fibre diameter, as fibre thinning and growth retardation occur reversibly with diminishing cysteine supply. Hair follicles express the amino acid transporter ASC, which will provide an active mechanism for the uptake of cysteine. Serine uptake by ORS cells is inhibited by excess cysteine, and is sodium-dependent, consistent with transport by system ASC.

Arginine can be oxidised to carbon dioxide by hair follicles, and its availability affects lactate production by follicles, reflecting changes in energy metabolism. However, most of the arginine taken up by follicles is incorporated into protein, primarily (60%) in the hair root sheaths, and partly (30%) in the hair fibre. Hair follicles express the gene for system y^+ . Uptake of arginine by ORS cells is inhibited by excess lysine or ornithine, which is consistent with transport by system y^+ , although the electrogenic regulation normally associated with y^+ was not clearly demonstrated.

The peptide transporters, PepT1 and HPT-1, and the putative transport subunit/activator encoded by rBAT, do not appear to be expressed by hair follicles. It is also possible that there is no active glutamate transporter present, since glutamate is transported into ORS cells by a markedly slower mechanism than arginine, cysteine or serine.

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"....even the very hairs of your head are all numbered."

(Luke 12:7)

CHAPTER I

GENERAL INTRODUCTION

1.1 Hair Follicles

Hair follicles are responsible for the production of hair, which although not essential to man for his survival, has several important functions, such as contributing to sexual attraction by its appearance. Its importance is more than cosmetic, although much distress is caused by hair loss or abnormality. However, hair also plays a role in insulation against heat loss, particularly from the head, and it contributes to sensory perception, while hair in the nose acts as a filter to trap small particles before they reach and obstruct the breathing passages. Hair may be classified as vellus or terminal. Vellus hairs are the fine, short, unpigmented hairs which cover most of the skin, while terminal hairs are the more prominent, coarser ones found on the scalp, beard, pubic and axilla sites. In this thesis, the focus of interest is human scalp hair, so only terminal hairs will be considered.

1.1.1 Hair Follicle Structure and Hair Growth

The structure of a hair follicle is illustrated diagrammatically in figure 1.1. The hair follicle consists of at least six layers of cells, all differentiating from a single germinative cell population, the hair matrix. The inner three layers form the actual hair fibre - the cortex, surrounded by the cuticle and containing the medulla. Surrounding the hair is the inner root sheath, which is overlaid with the outer root sheath, a layer which is continuous with the interfollicular epidermis of the skin.

Hair grows at approximately 0.3-0.35 mm per day (*Myers & Hamilton, 1951*),

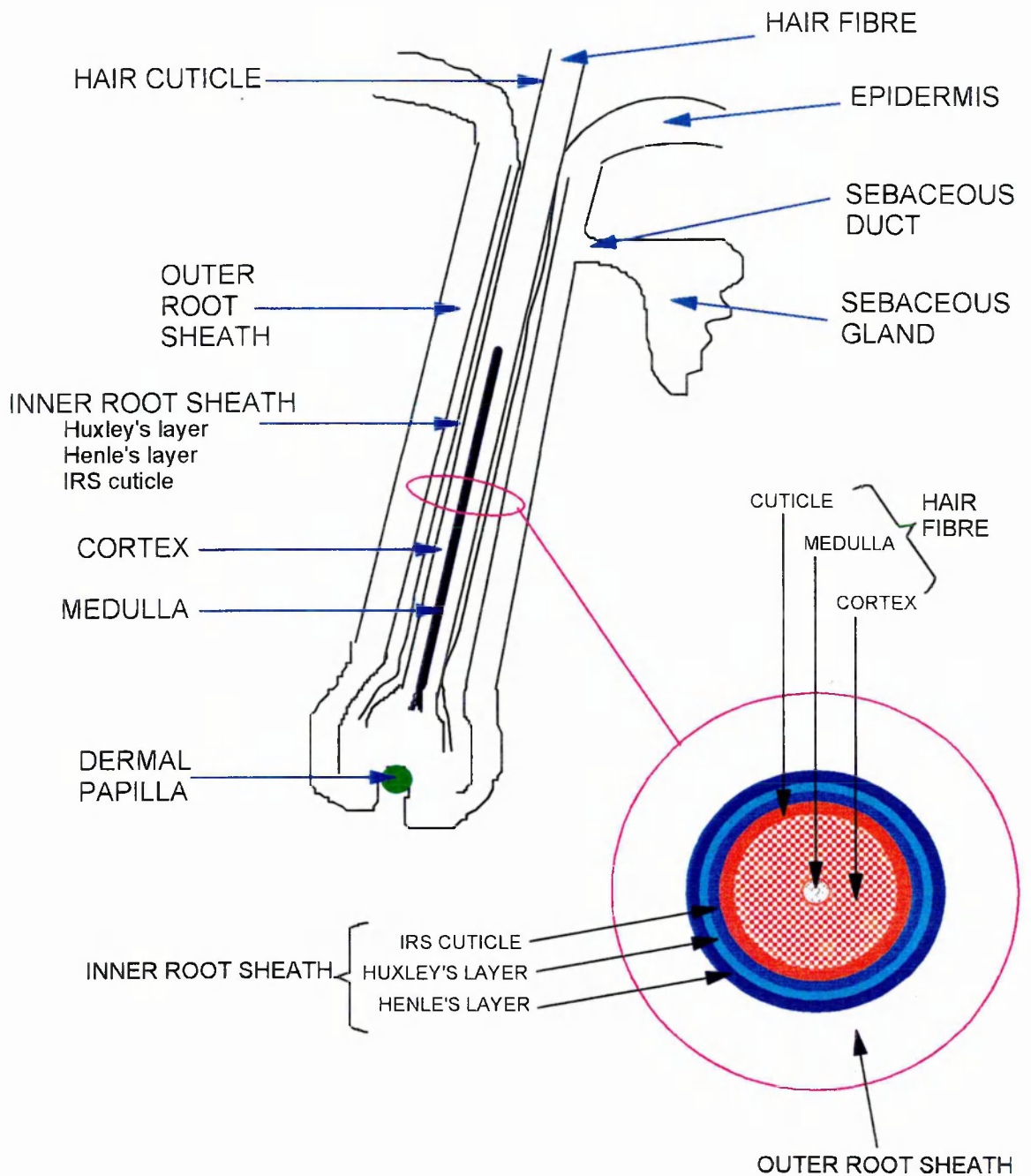
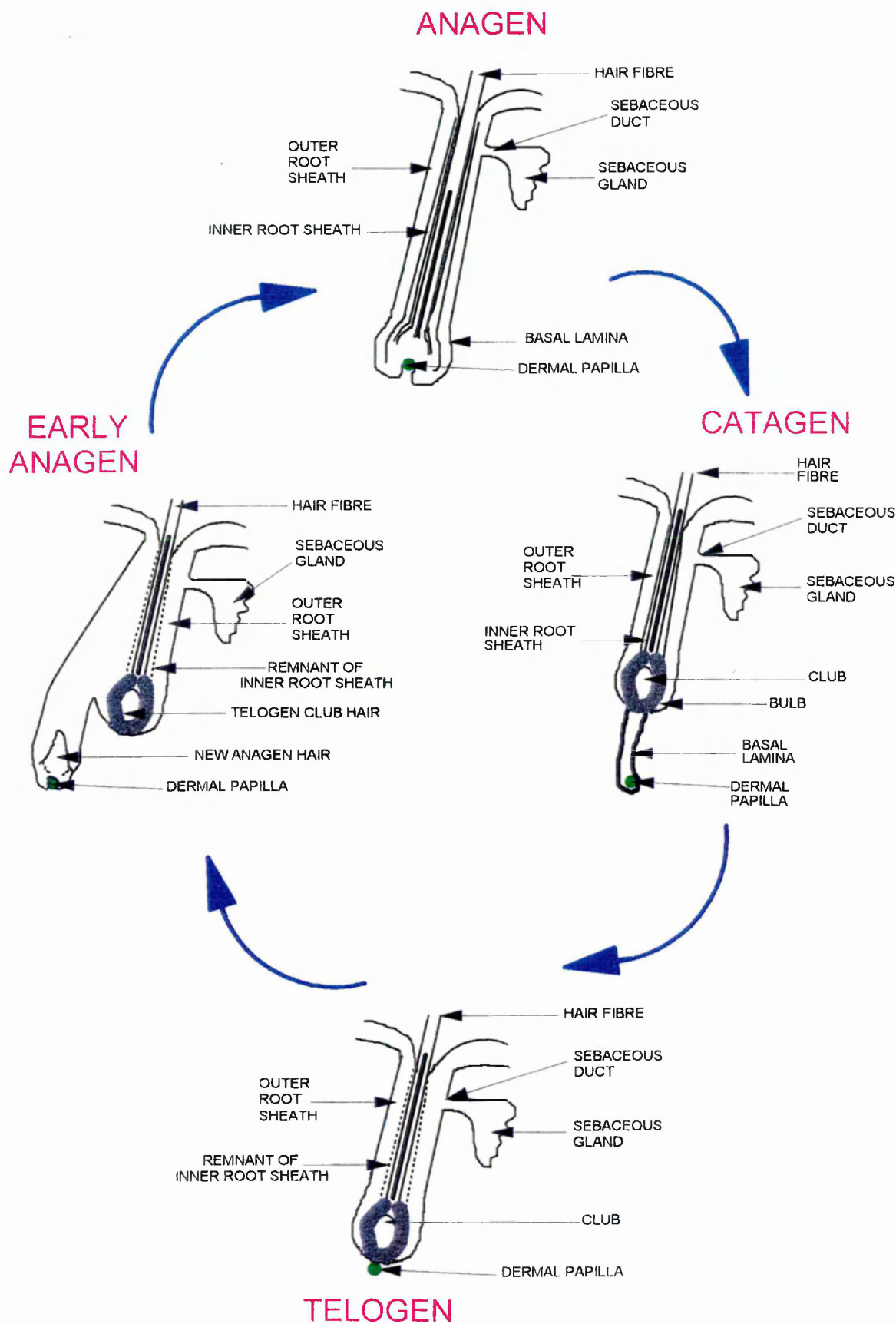


Figure 1.1: Structure of a Hair Follicle

and it is formed at the keratinizing region of the follicle, where the matrix cells differentiate and become keratinized (*Dover, 1988*). The keratin proteins undergo extensive cross-linking, to form an extremely tough, fibrous material. The medulla in human scalp hairs is often intermittent or absent (*Hashimoto, 1988*), and its function is unclear, although it may provide insulation, since it consists essentially of cells containing vacuoles filled with air. The hair cuticle is made up of a single layer (in humans) of overlapping, scale-like cells, which lie flat against the hair, pointing in the direction away from the bulb. The hair fibre cuticle has a protective role, but it undergoes weathering, such that the tip of the hair shows signs of wear relative to the newly-formed fibre close to the scalp. The role of the root sheaths in the follicle is not certain, although it is thought that the inner root sheath provides rigidity to control the shape and diameter of the emerging hair, and the outer root sheath is believed to contain pluripotential stem cells (*Cotsarelis et al., 1990*) that are important for hair growth but also play an important role in epidermal regeneration during wound healing. The follicle bulb contains blood capillaries and, most importantly, the dermal papilla, a group of specialised fibroblast cells essential for hair growth.

Hair growth is not continuous but occurs in cycles, controlled by the dermal papilla (*Oliver, 1966*). The active hair growth phase, known as anagen, lasts typically for about 5 years, and is followed by a brief regressive phase, catagen, during which the lower part of the follicle undergoes apoptosis (*Stenn et al., 1994*), giving rise to a much smaller resting or telogen follicle. The hair fibre remains in the follicle until a new cycle of hair formation begins, which results in the new hair fibre forming alongside the old hair, which is eventually shed. Figure 1.2 illustrates the hair growth cycle.

Figure 1.2: Hair Growth Cycle



1.1.2 Hair Follicle Metabolism and Nutrition

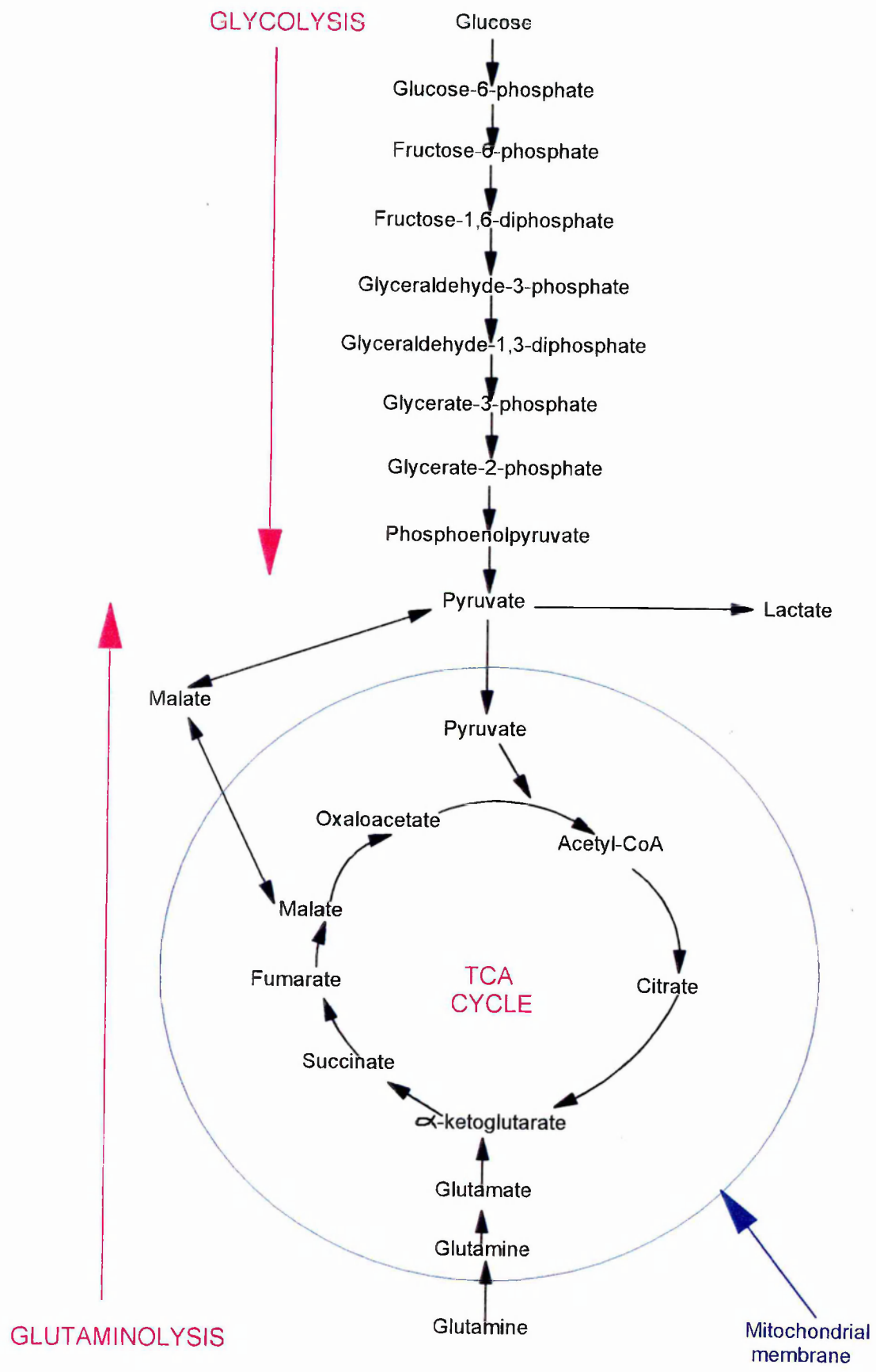
Hair follicles are rapidly growing skin appendages, and the dividing cells need a supply of energy as well as raw materials for structural growth. Hair follicles engage in aerobic glycolysis and glutaminolysis in order to provide for their energy requirements (*Philpott & Kealey, 1991*). Glucose is the major metabolic fuel utilised by hair follicles, but follicles may obtain almost as much energy from glutamine metabolism as from glucose (*Williams et al., 1993*). The majority of the glucose and glutamine taken up by hair follicles is only partially oxidised, in both cases culminating in lactate production. The pathways of aerobic glycolysis and glutaminolysis are shown in figure 1.3. Glutaminolysis was first defined as the linear pathway for the partial oxidation of glutamine to pyruvate by *McKeehan (1982)*, analogous to the incomplete oxidation of glucose to lactate.

One possible reason for the importance of glutamine as a substrate for hair follicle growth is the presence of an amide group on its side-chain, which would provide a nitrogen source, and could be involved in pH balance (*Kealey, personal communication*).

In mammalian cells, many amino acids are catabolised in energy-producing processes, involving the formation, directly or indirectly, of either a dicarboxylic acid intermediate of the tricarboxylic acid (TCA) cycle (*e.g.* glutamate is metabolised to α -ketoglutarate), or pyruvate (*e.g.* alanine undergoes a reversible transamination directly to pyruvate) or acetyl-CoA (*e.g.* tyrosine is converted to acetoacetyl-CoA and thence to acetyl-CoA). Thus, many amino acids, not just glutamine, may be metabolised to produce energy, as well as being available for incorporation into hair follicle proteins.

Hair is almost entirely protein, and amino acids are thus important substrates for

Figure 1.3: Metabolic Pathways of Glycolysis and Glutaminolysis



hair formation. As well as glutamine for energy metabolism, hair follicles require a supply of structural amino acids which can be cross-linked into proteins in the growing hair fibre. Hair consists of proteins called keratins, embedded in a matrix of intermediate filament associated proteins (IFAPs). Keratins are highly cross-linked by disulphide bonds and therefore cysteine is an important component of hair. In addition, the inner root sheath contains trichohyalin, which is rich in arginine and citrulline. Trichohyalin is an intermediate filament-associated protein that associates with keratin intermediate filaments of the IRS and medullary cells of the developing hair follicle. The exact role and function of trichohyalin is not yet known, but it has been postulated that it may serve as a cross-linking molecule, conferring a strong yet flexible scaffold-like structure to the tissue (*Lee et al., 1993*). Thus, trichohyalin may be of major importance in conferring structural function to the IRS, which will in turn determine the morphology of the hair fibre produced by the follicle. Trichohyalin consists of 30% arginine, and in addition many of its arginine molecules are converted to citrulline by the enzyme peptidylarginine deiminase (*Rogers et al., 1977*).

1.2 Outer Root Sheath Cells

As described in section 1.1.1., the outer root sheath (ORS) is the outermost layer of the hair follicle, and is continuous with the epidermis of the skin. Like the skin, the major cell-type of the ORS is the keratinocyte. In most hair follicles the ORS is highly vascularised and is likely to be the first point of contact between amino acids in the blood and the hair follicle. There is evidence to suggest that the ORS may actively transport amino acids to the keratinising region of the hair fibre (*M.Philpott, personal communication*). Keratinocytes from different portions of plucked hairs have

been examined to compare their proliferative capacity (*Moll, 1995*), and it was found that both the colony-forming potential and the life-span were greatest for keratinocytes from the middle portion of the hair.

1.3 Amino Acid Transport

As discussed above, amino acids are important substrates for both energy metabolism in follicles and for hair fibre formation. The hair follicle is surrounded by blood vessels, but the cells need to have a mechanism for acquiring the necessary amino acids from the blood. Amino acid transport across the plasma membrane is, therefore, a process of fundamental physiological importance, regulating the flow of these nutrients into cells. It is mediated by specific proteins that recognise, bind and transport amino acids from the extracellular medium into the cell or *vice versa*. Various transport systems have been identified with overlapping specificities, but until recently little was known about their structure and molecular properties. Table 1.1 describes the major amino acid transport systems identified in mammalian tissues so far. Since many transport systems exist, with redundancy of substrate specificity, a particular amino acid will be a substrate for more than one system.

Amino acid transporters may be classified by their ion-dependence, as well as by their substrate-specificity. Some transporters use chemical energy from ATP directly (*primary active transport*) while others couple transport to the movement of another species, usually Na^+ or H^+ , down its electrochemical gradient (*secondary active transport*). Mediated transport, which does not require energy, is termed *facilitated diffusion*, and like active transport, it involves specific recognition of certain amino acids by the transport protein, and is saturable but not concentrative.

Table 1.1: Mammalian Amino Acid Transporters

SUBSTRATE SPECIFICITY		REGULATION	DISTRIBUTION
<u>Systems for neutral amino acids</u>			
Sodium-dependent:			
A	most aliphatic amino acids, <i>eg.</i> ala, gly, pro; MeAIB	starvation, hormones, growth factors, pH	widespread
ASC	<i>eg.</i> ala, ser, cysteine, thr; not N-methylated	not usually inducible; <i>trans</i> -stimulated	widespread
N	Gln, his, asn	sensitive to pH	liver, red blood cells (variant Nm in skeletal muscle)
Gly	Gly, sarcosine	Cl ⁻ dependent	liver, brain, red blood cells
B ^o	broad (includes branched- chain & aromatic, unlike ASC)	?	brush-borders (kidney, gut)
B ^{o,+}	broad:- most neutral & dibasic amino acids	?	Blastocysts, fibroblasts, Xenopus oocytes
IMINO	(hydroxy)prolines, N-methyl- glycines; interacts with MeAIB	?	brush-border membranes (intestine)
Sodium-independent:			
L	includes bulky amino acids, <i>eg.</i> ile, phe, met, val, cys, leu	not usually inducible; <i>trans</i> -stimulated- heteroexchange occurs	widespread
asc	As system ASC	As system ASC	red blood cells of some species (<i>eg.</i> horse)
b ^{o,+}	like B ^{o,+} , but limited by branch structures	electrogenic (<i>ie.</i> membrane potential)	Blastocysts, brush-border membranes

Table 1.1 (continued): Mammalian Amino Acid Transporters

SUBSTRATE SPECIFICITY	REGULATION	DISTRIBUTION
<u>Systems for cationic amino acids</u>		
(Sodium-independent)		
y^+ lys, arg, his	electrogenic	widespread (variants known)
y^+L dibasic & neutral amino acids	needs Na^+ for neutral a.a. transport	lymphocytes, red blood cells
<u>Systems for anionic amino acids</u>		
Sodium-dependent:		
X_{AG}^- L-glu, L-asg, D-asg (high affinity)	electrogenic; needs K^+ , and $> 1 Na^+$ per a.a.	widespread
Sodium-independent:		
x_G^- glu & analogues, largely excluding asp	?	neurones
x_c^- glu & cystine	electroneutral	liver, fibroblasts

(Recent reviews of mammalian amino acid transport include *Bertran et al., 1994* and *McGivan & Pastor-Anglada, 1994*.)

The general process of membrane transport is illustrated in figure 1.4. Exchange of amino acids by the transport protein may also occur (*e.g. Coady et al., 1994*), although only unidirectional flux is illustrated in this diagram.

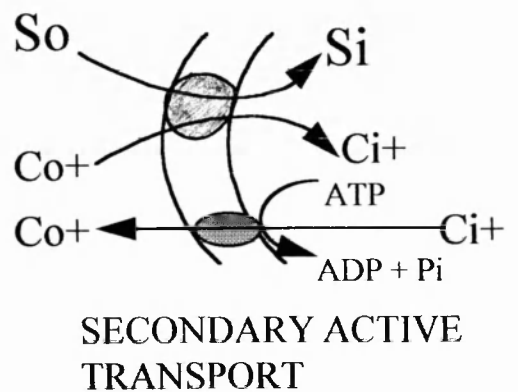
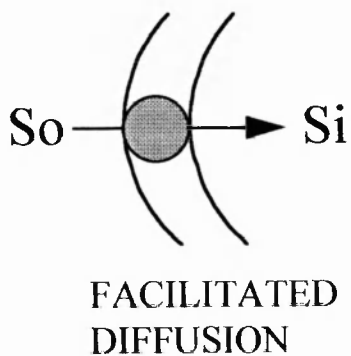
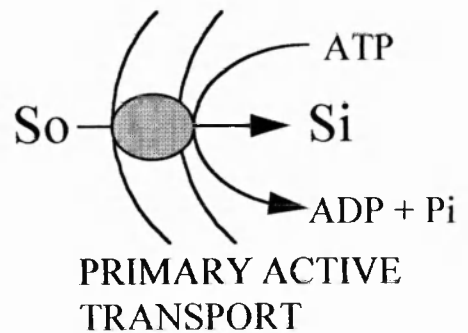
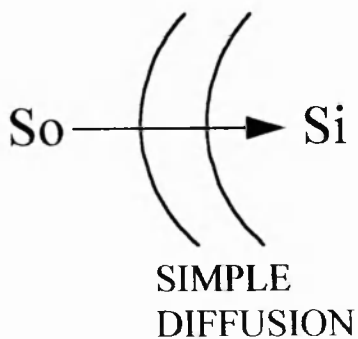
Transport is a process which can be regulated by cells, in order to match the uptake or efflux of an amino acid with its requirements. Regulation of transport may be mediated by a number of molecules, such as hormones or growth factors, as well as by parameters such as pH, osmolarity and membrane potential. For example, glucagon and epidermal growth factor are both known to up-regulate System A (*Shotwell et al., 1983; Moule & McGivan, 1987*). Some transporters, *e.g.* System A, are subject to *adaptive regulation*, where the synthesis of the transporter protein is regulated in response to substrate requirements and availability. Another important aspect of transport is its kinetic characteristics, since this will determine parameters such as saturation and maximum rates of transport.

In addition, one substrate may affect the influx or efflux of another by competing or stimulating at the transport site (*cis-inhibition or cis-stimulation*), or by interacting on the opposite side of the membrane to affect the availability of the transport sites in the proper orientation for transport (*trans-stimulation or trans-inhibition*). *Trans*-stimulation occurs if the transporter-substrate complex shifts to the opposite-facing conformation faster than the free carrier; *trans*-inhibition results if the loaded complex reorientates more slowly than the free carrier. This interaction between substrates and other solutes is an important consideration as it has implications for the follicular uptake of important hair nutrients. An understanding of the natural regulatory mechanisms present may offer opportunities for us to modify the supply of important amino acids to hair follicles.

Figure 1.4: Transport across cell membranes.

So = substrate outside cell, Si = substrate inside cell,

Co⁺ = cation outside cell, Ci⁺ = cation inside cell.



1.3.1 Na⁺-Dependent Transport Systems

These are shown in table 1.1, but below is some additional detail about the transporters which are of importance in this thesis.

System A

Characterised by its Na⁺-dependence and its tolerance of N-methylated substrates, system A transports neutral amino acids, preferentially short, straight-chain ones such as alanine. It is subject to significant regulation by amino acid availability (starvation-induced transport), hormonal regulation and *trans*-inhibition (Shotwell et al., 1983).

System ASC

System ASC transports neutral amino acids, in particular cysteine, serine, alanine and threonine, in a sodium-dependent manner (*Shafqat et al., 1993*). It does not recognise N-methylated amino acid derivatives, and is relatively insensitive to adaptive regulation and hormonal control.

1.3.2 Na⁺-Independent Transport Systems

System y⁺

The sodium-independent System y⁺ transports basic amino acids such as arginine, and it is affected by membrane potential (*White, 1985*). It is insensitive to pH changes.

1.3.3 Other Amino Acid Transport Systems

A family of possible specific activators or subunits of amino acid transporters has been identified, including rBAT which seems to play a role in the transport of both cationic and neutral amino acids (*Palacín, 1994*). They do not appear to be actual transporters, because their predicted structure has only a single transmembrane domain,

as described in section 1.3.4.

1.3.4 Molecular Biology of Amino Acid Transport Systems

Using the method of expression cloning, a limited number of amino acid transporters has now been cloned, enabling the study of transport regulation at the transcriptional level. In this technique, mRNA (obtained from DNA libraries) is injected into *Xenopus laevis* oocytes. If subsequent expression of the mRNA gives rise to a measurable effect, *e.g.* an increase in arginine transport, a function can be assigned to the mRNA. The transport protein is not identified directly, but its structure is inferred from the sequence of the putative transporter gene. Therefore, functional studies are still necessary to confirm the characteristics of the protein. One problem with the method of expression cloning is that the oocyte possesses a number of native amino acid transporters, so a high level of background amino acid transport is present. It is also possible that the injected RNA is activating native transporters, *i.e.* that it encodes a transport regulator, rather than an actual transport protein - this may be implied by the predicted protein structure, *e.g.* in the case of rBAT, which is only a single transmembrane protein, whereas transporters are expected to consist of several such domains. Another problem is the possibility that not all mammalian transport proteins may be able to be expressed in oocytes, and correct glycosylation of the protein may not occur. Mammalian cells such as monkey cells have occasionally been used as alternative expression vectors (*Mellon et al., 1981*), but expression of the injected RNA is only transient.

The first mammalian amino acid transporter to be identified in this way was system y^+ (mCAT1). It was originally identified as the mouse ecotropic virus receptor

(Kim *et al.*, 1991; Wang *et al.*, 1991). When mRNA encoding this receptor was injected into *Xenopus* oocytes, uptake of basic amino acids was increased, and the properties of this additional uptake were consistent with those of the y^+ transporter. In addition, the mRNA for CAT1 had the same tissue distribution as system y^+ .

Expression cloning has also been used to identify the neutral amino acid transporter ASC, as clone ASCT1 (Aririza *et al.*, 1993). Expression of this in *Xenopus* oocytes results in the Na^+ -dependent uptake of L-alanine, L-cysteine and L-serine. By probing a cDNA library with a cDNA probe from human brain tissue which showed homology with an *Escherichia coli* glutamate transporter, a similar clone was obtained, SATT (Shafqat *et al.*, 1993). Expression of SATT in HeLa cells resulted in system ASC-like transport activity, but there was no uptake of L-cysteine, so SATT is unlikely to be system ASC.

EAAC1 has been cloned from rabbit small intestine (Kanai & Hediger, 1992). This encodes a glutamate transporter which has the characteristics of system X_{AG}^- . It shares significant amino acid sequence similarity with the putative SATT protein sequence (Shafqat *et al.*, 1993).

The SAAT1 gene was cloned and was thought to be system A, as it induced Na^+ -dependent MeAIB transport in transfected COS cells (Kong *et al.*, 1993). However, more recent work (Plakidou-Dymock *et al.*, 1994) suggests it is more likely to be related to system B°. Other methods of investigating system A activity have been applied, too. Gene amplification has been used to obtain enhanced activity in NBL-1 cells (Felipe *et al.*, 1992). System A is subject to *adaptive regulation*, where the synthesis of the transporter protein is regulated in response to the substrate requirements and availability. Therefore, by culturing cells in the absence of amino acids, increased

expression of system A is obtained. Tritium suicide selection has also been used to study system A (*Dantzig et al., 1982*). Cells are grown in medium containing ^3H -labelled amino acid. Normal cells accumulate radiolabelled amino acid and are killed by the radioactive decay, but cells with mutant transporters accumulate less and survive.

Transporters for peptides have also been identified, including HPT-1 (*Dantzig et al., 1994*) and PepT1 (*Fei et al., 1994*). PepT1 is a proton/peptide cotransporter of broad substrate specificity found in the apical membrane of epithelial cells. This was the first proton-coupled organic solute carrier reported in vertebrates (*Ganapathy et al., 1994*).

In addition, a family of possible specific activators or subunits of amino acid transporters has been identified, including rBAT which seems to play a role in the transport of both cationic and neutral amino acids (*Palacín, 1994*). When rBAT RNA was injected into *Xenopus laevis* oocytes, a system b^0+ -like transport activity was induced (*Bertran et al., 1992; Tate et al., 1992*), *i.e.* a high-affinity sodium-independent uptake of cationic and neutral amino acids. Unlike system b^0+ , however, rBAT induces cystine transport. Because the protein is predicted to have only one or four transmembrane domain(s), it is unlikely to represent an entire transporter protein, as it cannot form a pore, so it may be a subunit or a regulator of another amino acid transporter. An example of a single helix protein being a transporter subunit is the recently-cloned MinK potassium channel (*Hoshi & Zagotta, 1993*), which aggregates to form a channel consisting of several subunits. Thus, if this rBAT is expressed in hair follicles, it may play a role in regulating cystine transport.

1.4 Aims of the studies comprising this thesis

The aims of the studies described in this thesis were to study hair follicle nutrition and metabolism in order to:-

1. Identify amino acids important for maintaining normal hair growth *in vitro*
2. Determine regions in the follicle where different amino acids are likely to be most important
3. Determine which amino acid transporters are expressed in human hair follicles in comparison with keratinocytes derived from the follicle by explant culture
4. Measure amino acid transport into follicle-derived cultured keratinocytes.

CHAPTER II

METHODS

2.1 Materials and Media

Hair follicles were maintained in William's E medium (supplied by GIBCO) supplemented with 10 μ g/ml insulin, 10 ng/ml hydrocortisone, 2 mM glutamine and 50 μ g/ml penicillin and streptomycin (all from Sigma).

For experiments where a range of amino acid concentrations was required, modified William's E medium, "Medium 1" (GIBCO), which was prepared without the amino acids methionine, cysteine, cystine, lysine, arginine, isoleucine and glutamate was used. On the day of follicle culture, additions were made to this medium such that the final concentrations of the amino acids were those which occur in normal William's E medium, except for the amino acid of interest. Amino acids for medium supplementation and for transport studies were obtained from Sigma. Radiolabelled amino acids were from Amersham.

Outer root sheath cells were maintained in Clonetics' Keratinocyte Growth Medium (KGM) supplemented with insulin, epidermal growth factor (EGF), bovine pituitary extract (BPE) and hydrocortisone (Clonetics standard supplements).

LSDM3 cells were cultured in Dulbecco's Minimum Essential Medium (DMEM) containing Glutamax[®] (GIBCO), supplemented with 10% foetal calf serum (Sigma).

2.2 Isolation and Culture of Human Hair Follicles

Follicles were isolated from human scalp skin obtained from facelift surgery, by the method of *Philpott et al.*, 1990. Briefly, the skin was cut into small pieces (1 x 0.5

cm) and using a scalpel the subcutaneous fat, containing the follicle bulbs, was separated from the epidermis and dermis. Follicles were then removed from the fat under a dissecting microscope using watchmakers forceps to pluck the follicle from the fat. Isolated follicles were placed into William's E medium. If necessary, the follicles were trimmed to remove the tip of the cut end, if it was damaged during isolation, or to ensure that follicles were of similar lengths. Follicles were cultured in 24 well multi-well plates containing 1 follicle per well in 1ml of medium. Follicles were maintained in an atmosphere of 5 % CO₂ / 95 % air, at 37°C, and the medium was changed every 3 days.

2.3 Measurement of Amino Acid Uptake into isolated Hair Follicles.

Follicles were cultured initially in William's E medium for 18 hours. Four batches of 5 follicles in 100µl of William's E medium containing L-[¹⁴C-U]-arginine (specific activity 74 MBq/mmol), were incubated at 37°C, shaking in an atmosphere of 5 % CO₂ / 95 % air. At various time-points up to 5 hours, 5 follicles were removed from the radioactive medium and washed with 3 x 1ml of phosphate buffered saline (PBS) supplemented with 10mM cold arginine, to displace non-covalently bound ¹⁴C-arginine. The follicles were dissolved in 90% Soluene (60°C for 2h), then counted with 18ml Hionicfluor scintillant (supplied by Canberra Packard). Unlabelled follicles were dissolved in Soluene in the same way and counted as a blank.

2.4 Localisation of Amino Acid Uptake in isolated Hair Follicles

Follicles were incubated at 10 follicles/ml, at 37°C in an atmosphere of 5 % CO₂ / 95 % air in William's E medium containing L-[¹⁴C-U]-arginine (specific activity 74

MBq/mmol), for 24 hours. The follicles were washed x 3 with warm unlabelled William's E medium, then plated out and cultured in unlabelled control William's E medium, for a cold chase period. Five follicles were removed immediately, five more after 24 hours chasing, and five more after 4 days chasing, and these were all fixed in 10% neutral buffered formalin to enable histology and autoradiography to be carried out. After the 4 days chasing, the remaining growing follicles were dissected into connective tissue sheath (CTS), root sheaths (outer and inner) and hair fibres. The corresponding parts of the follicles were pooled, dissolved in 90% Soluene and counted using HionicFluor scintillant. The follicles that had been fixed were processed for paraffin sectioning by the laboratory histologist. Serial sections were cut at 4 μ m and every tenth section stained with haematoxylin and eosin (H & E). Suitable sections for autoradiography were selected, coated with Ilford K5 emulsion and exposed at 4°C for between 3 days and one week. The emulsion was developed at 20°C for 3 min. in Kodak D19, rinsed in 2% acetic acid and fixed in Ilford Hypan (1:4 dilution in doubly distilled H₂O) for 6 min. The sections were counterstained with haematoxylin, dehydrated, cleared and mounted in XAM synthetic resin mountant.

2.5 Measurement of Rate of Protein Synthesis by Hair Follicles

The use of leucine for measuring protein synthesis was validated (see Chapter III). All experiments were carried out in triplicate samples of 5 follicles in 100 μ l of Williams' E medium or Medium 1 with one of the chosen concentrations of the amino acid of interest, containing [U-¹⁴C]leucine (specific activity 74 MBq/mmol). Follicles were incubated for 3 hours at 37°C, shaking in an atmosphere of 5% CO₂ / 95% air. After incubation, the follicles were removed from the radioactive media, and washed

with 3 x 1ml of phosphate buffered saline (PBS) supplemented with 10mM cold leucine, to displace non-covalently bound ^{14}C -leucine. Follicles were then homogenised in 0.1ml of ice-cold 0.1M K_2EDTA , pH 12.3, in a hand-held glass/glass homogeniser. The homogenate was washed out of the homogeniser with 0.9ml K_2EDTA , and then left at 4°C for 30 minutes to solubilise the protein. Cell debris was removed by microcentrifugation for 2 minutes at 13000rpm (9500g), and 0.5ml of ice-cold 15% w/v trichloroacetic acid (TCA) was added. This was left at 4°C for 20 min. to precipitate the protein. The precipitate was collected, and washed with 4 x 5ml of 15% TCA, under vacuum onto Whatman GF/C (2.4 cm) filters, which had been pre-washed with PBS supplemented with 10mM cold leucine. Finally, the filters were dried and their radioactivity determined by liquid scintillation spectrometry. The cell debris was solubilised in 0.5ml of Soluene, and found to contain less than 15% of incorporated radioactivity.

2.6 Measurement of Amino Acid Oxidation to Carbon Dioxide by isolated Hair Follicles

Follicles were incubated at 5 follicles/100 μl , shaking at 37°C in an atmosphere of 5% CO_2 / 95% air in William's E medium containing L- ^{14}C -U]-arginine (specific activity 74 MBq/mmol). Samples without follicles were also set up as controls, to measure non-follicular release of $^{14}\text{CO}_2$. Incubations were carried out in open eppendorf tubes placed in glass scintillation vials sealed with rubber subaseals. After 0, 1, 3 and 6 hours, 500 μl benzethonium hydroxide was injected into 3 sample and 3 control scintillation vials, and 100 μl of 25% v/v perchloric acid (PCA) into the eppendorf tubes. The benzethonium hydroxide absorbs the CO_2 produced, while the PCA stops

substrate oxidation and releases the CO₂. The tubes were left shaking for 19 hours after the final time-point, then the seals and eppendorfs were removed from the scintillation vials. Hionicfluor was added, and the vials counted.

Results were expressed as pmoles arginine oxidation / follicle, using the formula:

$$\text{amount of arg oxidised} = \frac{{}^{14}\text{CO}_2 \text{ formed (dpm)}}{\text{specific activity } {}^{14}\text{C-arginine}}$$

2.7 Measurement of Lactate Production by Hair Follicles

Follicles were incubated in Williams' E medium containing either 0, 0.287mM, 0.574mM or 1.435mM arginine at 37°C, in an atmosphere of 5% CO₂ / 95% air. At various time-points, duplicate or triplicate samples of 5 follicles were removed, and the medium collected and assayed for lactate content, to measure the amount of lactate produced by the follicles in measured time-periods or at the different arginine concentrations used. Lactate production was determined using the method of *Gutmann & Wahlefeld (1974)*. Lactate dehydrogenase (19U/ml) catalyses the conversion of lactate to pyruvate in the presence of glycine (0.43M) and hydrazine (0.34M) at pH 9 and 2.75mM NAD:



The alkaline hydrazine buffer traps pyruvate as pyruvate hydrazone and buffers protons as they are produced, such that the reverse reaction is inhibited. NADH formation was measured by the increase in absorbance at 340nm, after allowing the reaction to run to

completion for 35 minutes at 25°C. Lactate will react with an excess of NAD in a 1:1 ratio, so the NADH produced equals the amount of lactate initially present. Using standards, a calibration curve was created (figure 2), which was used in conjunction with the Beer-Lambert law to estimate the lactate concentration.

Beer-Lambert law:

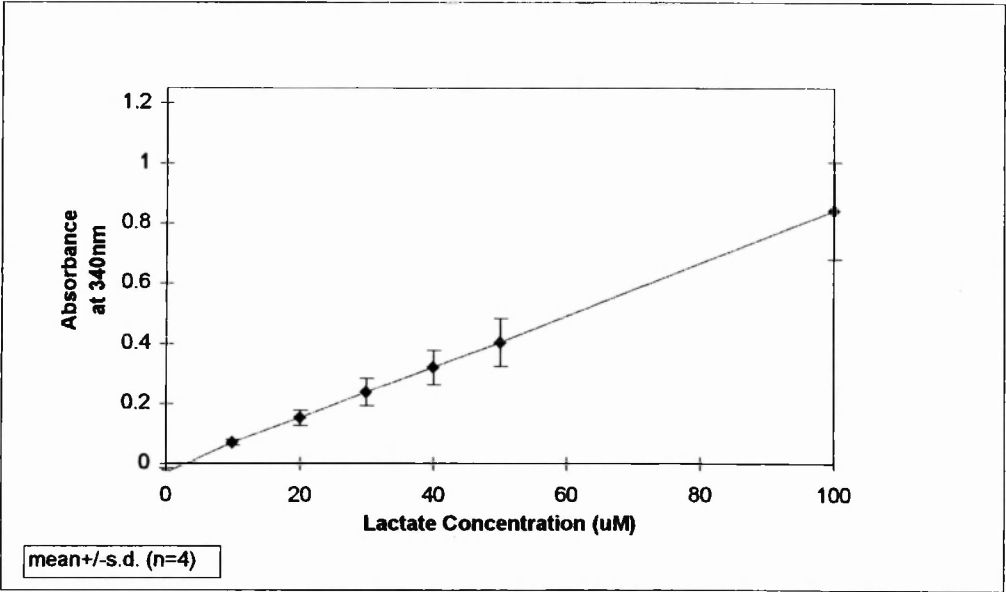
$$A = \alpha cl \quad \text{where} \quad \begin{array}{l} A = \text{absorbance} \\ \alpha \text{ for a 1M solution of NADH is } 6.22 \times 10^3 \text{ cm}^{-1} \\ c = \text{concentration} \\ l = \text{path length (1 cm)} \end{array}$$

2.8 Isolation and Culture of Outer Root Sheath Cells

Outer root sheath cells were obtained from hair follicles by explant culture. After isolation of hair follicles from facelift skin as described in 2.2, the bulbs were removed with a scalpel, and the follicles exposed to 1 mg/ml trypsin-EDTA (Sigma) for 30 minutes, then 1 mg/ml soybean trypsin inhibitor for 10 minutes. They were then transferred into culture flasks containing Clonetics' Keratinocyte Growth Medium (KGM) without serum or antibiotics. This method is confirmed by the findings of *Moll, 1995*, that the part of the follicle used here has the greatest colony-forming ability and the longest life-span in culture, and also that ORS cells have longer life spans in KGM than on feeder layers.

After reaching sub-confluence, cells were passaged as follows. The medium was removed, the cells washed with phosphate buffered saline (PBS), and trypsin-EDTA added (2ml for a 25cm² flask). The cells were incubated at 37°C for about 10min until they were detached from the flask upon knocking the flask sharply. Trypsin inhibitor was added, and the cells transferred to a Universal tube, washing out the flask with

Figure 2: Calibration Curve for Lactate



The absorbance at 340 nm was measured over a range of lactate concentrations, as described in the text. The graph shows the mean +/- S.D. for n=4 experiments.

KGM. After spinning at 1000rpm for 5 min, the cell pellet was resuspended in fresh KGM and plated out at a density of approximately 10^4 cells per cm^2 . Cells were passaged either into bigger flasks to gain increased numbers of cells, or directly into 12-well tissue culture plates. When sufficient cells were obtained for 2 or more plates, cells were passaged and seeded into plates at $3-4 \times 10^4$ cells per well, in 1 ml of KGM. The medium was changed every 2-3 days, and transport experiments were carried out when the cells reached sub-confluence, typically within 7 days. Cells were maintained in an atmosphere of 5% CO_2 / 95% air, at 37°C , and the medium was changed every 2-3 days.

2.9 Culture of LSDM3 Cells

The study of hair follicles can be done more simply at the cellular level for some investigations, such as the measurement of amino acid transport. As ORS cells are relatively slow-growing, method development can be done on a more rapidly-proliferating cell-line instead. LSDM3 is an immortalised human keratinocyte cell-line derived from a tumour, kindly donated by Imperial Cancer Research Technology Ltd. LSDM3 cells were cultured in Dulbecco's Minimum Essential Medium (DMEM) containing Glutamax[®] (GIBCO), and supplemented with 10% foetal calf serum (Sigma). They were seeded into 12-well plates at $3-4 \times 10^4$ cells per well, in a volume of 1 ml medium, and used at sub-confluence in the same way as ORS cells.

2.10 Measurement of Amino Acid Transport by Cells

Medium was removed by aspiration, and replaced with medium containing ^3H -leucine at 37 KBq / 0.5 ml /well (specific activity 148 MBq / mmol), for about 1 hour.

This labelled all proteins synthesised, to give a comparison of cell numbers per well (validated in Chapter V). The plates were then equilibrated to room temperature (about 22°C) for 5-10 minutes. The cells, which had formed sub-confluent monolayers adhering to the plate, were washed rapidly 5 times with 0.5 ml/well of saline buffer (10 mM MOPS, 5 mM glucose, 5 mM KCl and 145 mM NaCl, pH 7.4). To each well was added 0.5 ml of the saline buffer containing the amino acid of interest, at 3.7 KBq / 0.5ml of ¹⁴C-labelled amino acid plus a known concentration of unlabelled amino acid. At specific time-points, the solution was removed by aspiration, and the cells washed 6 times with ice-cold "stop solution" (107 mM MgCl₂, 10 mM MOPS, pH 7.4). The cold temperature, competing divalent cations and the dilution effect combine to stop amino acid transport. The cells were then ruptured by adding 0.5 ml of 0.5 M NaOH per well for at least 20 min. The contents of the ruptured cells were collected together with 3 x 0.5 ml water washes, and counted with 18 ml HionicFluor scintillant, by dual-label scintillation counting. The uptake of amino acid was calculated by:

$$\text{Uptake} = \frac{\text{dpm of sample} \times \text{amount of unlabelled amino acid present}}{\text{dpm added}}$$

Results were expressed as a ratio of amino acid uptake / leucine incorporation.

2.10.1 Na⁺-Dependence of Amino Acid Transport

As discussed in Chapter I, a number of amino acid transport systems in other cell types have been shown to function only in the presence of Na⁺ ions. Thus, by measuring the rate of transport in the presence and in the absence of Na⁺, the difference

between these rates will give the Na^+ -dependent rate of uptake. NaCl in the saline buffer was replaced by N-methyl-D-glucamine chloride (NMDG.Cl) to give Na-free saline, which was used in the same way as the saline buffer in method 2.5, i.e. to wash the cells and then to add with the amino acid being transported.

2.10.2 Regulation of Amino Acid Transport

The putative inhibitor or stimulator as described in the results was added to the saline buffer containing the amino acid of interest. When Minoxidil was tested for its effects on amino acid transport, it was also added to the ^3H -leucine-containing medium for the protein-labelling incubation of the cells prior to the transport measurements, as well as being added to the saline buffer.

2.11 Measurement of Cell Volume

Cells were incubated for 5 minutes (to allow equilibration) with a mixture of ^3H -sucrose (specific activity 148 MBq / mmol) and ^{14}C -2-deoxyglucose (specific activity 14.8 MBq / mmol). Sucrose cannot cross the cell membrane, and so it distributes within the extracellular spaces, while 2-deoxyglucose equilibrates across the cell membrane to fill both the intra- and the extra-cellular spaces. Thus, the difference between the ^3H and ^{14}C space gives a measure of the intracellular volume (*Ellory, 1982*). The extracellular space marker, in this case sucrose, is used at the higher specific activity, as it will ultimately represent a smaller value than the total volume, therefore it is tritiated, as ^3H counting is less efficient than ^{14}C , and the counts need to be higher for accuracy. A count of cell number, using the Coulter counter, enables the intracellular volume per well to be divided by the number of cells per well, to give an estimate of the volume

of a single cell.

2.12 Molecular Biology

2.12.1 Total RNA Isolation

Cells were harvested from culture flasks by washing with PBS, then adding guanidinium isothiocyanate (GISO) solution (4M GISO, 0.5%w/v sodium dodecylsulphate (SDS), 25mM sodium citrate, pH 7 with 1M NaOH) at 0.5ml per 10^6 cells. The cells were scraped off the flask into the GISO on ice using a cell scraper.

Hair follicles were isolated as described in 2.2 then homogenised in GISO, 60 follicles per 0.5ml GISO. Cells or follicles in GISO were stored at 4°C.

Isolation of total RNA from cells or follicles was carried out using a micro-scale total RNA separator kit from Clontech. This is a modification of the *Chomczynski & Sacchi (1987)* method, and involves ethanol precipitation from the GISO followed by extraction with phenol at reduced pH, to remove protein and DNA, followed by isopropanol precipitation to concentrate the RNA. The RNA obtained was resuspended in diethyl pyrocarbonate (DEPC)-treated water and stored at -70°C as an ethanol precipitate (1 x volume of RNA suspension plus 2.5 x volume of absolute ethanol and 0.1 x volume of 5M ammonium acetate).

2.12.2 Quantification of RNA or DNA

RNA in solution in DEPC-treated water was quantified by measuring the optical density at 260nm, using H₂O as the reference blank. At 260nm, 1.0 absorbance unit = 40 µg/ml RNA. For 2µl of RNA solution in 500µl DEPC-H₂O, the dilution factor is

x250, so:

$$\text{Amount of RNA} = A_{260} \times 250 \times 40 \mu\text{g/ml} = A_{260} \times 10 \mu\text{g}/\mu\text{l}$$

In addition, electrophoresis of the RNA was carried out. Both methods indicate the purity and quality of the RNA. The ratio of A_{260}/A_{280} should be about 2 for pure RNA. The appearance of RNA on a gel, visualised under ultraviolet light, is a characteristic pattern of bands, which if smeared would suggest that the RNA was degraded. 28S and 18S rRNA and tRNA bands should be visible, and the 28S band is normally twice as bright as the 18S band. (See figure 4.3).

To quantify the PCR products (*i.e.* DNA) after gel-purification, prior to sequencing, the DNA sample was subjected to electrophoresis on an agarose gel alongside some mass markers (Life Technologies, Inc.), whose brightness corresponds to the amount of DNA present.

2.12.3 Agarose Gel Electrophoresis

To run RNA on a gel, a 1% agarose gel was made by dissolving 0.5g agarose (RNase-free) in 50ml of electrophoresis buffer (10.903 g/l Tris, 5.565 g/l boric acid, 0.372 g/l EDTA) by heating, and then adding 3 μ l of ethidium bromide, mixing, and pouring the gel mixture into a gel holder with comb and end-plates in place. To run PCR products (*i.e.* DNA) on a gel, a 1.5% agarose gel was made, using 0.75g agarose in 50ml ELFO buffer, and treating it in the same way. When the gel had set, the comb and end-plates were removed and 50ml ELFO buffer was poured over it. 3 μ l of RNA, or 12.5 μ l of DNA, was mixed with 10 μ l of Orange G dye (Gurr), and loaded into the wells on the gel using a Gilson pipette. The gel was run from negative to positive at 100 V for 30 minutes. It was observed under an ultraviolet lamp.

2.12.4 DNase Treatment of RNA

To 4.5µg RNA was added 20µl 5xTranscription buffer (Promega), 12.5µl dithiothreitol (dTT, 10mM) (Promega), 25µl RQI DNase I (Promega 1U/ml) and 36.5µl DEPC H₂O. After vortexing then microcentrifuging for 10sec, the RNA mixture was incubated at 37°C for 20min, then transferred to ice and 250µl ethanol plus 6µl ammonium acetate (5mM stock) added. This was kept at -20°C for at least 1 hour to precipitate the RNA. This was then spun down at 14000rpm (12000g) for 20min at 4°C, and the RNA pellet resuspended in DEPC H₂O.

2.12.5 RT-PCR Oligonucleotides

The primers used for PCR were chosen using the computer programme *PrimerSelect* of the DNA Star package on CD ROM. Features of successful primers (Saiki, 1989) tend to include:

- length of about 20-30 bases
- minimal secondary structure
- not complementary to each other
- at least 50% GC content

There are 3 known genes for the y⁺ transporter, and the one probed for here was the retroviral receptor mCAT1 (Yoshimoto *et al.*, 1991).

The primers used for mCAT1 were as follows:

Upper Primer (19-mer): 5' CCGCCGGCTTGGATTCTGA 3'

Lower Primer (20-mer): 5' GGGGACCCGAGCACCAAAC 3'

The SATT gene for ASC was cloned by *Shafqat et al.* in 1993. The first primers were designed by reference to this paper, without the aid of the computer programme:

Primer ASC1 (18-mer): 5' ACACGAAAAAACCCATTC 3'

Primer ASC2 (17-mer): 5' CGCCTGCTGCTCCAACA 3'

Primer ASC3 (18-mer): 5' CCCATTTGCGACAGATTT 3'

These were used as pairs 1&2 and 2&3, looking at two portions of the gene in order to distinguish between the SATT gene and the closely related EAA glutamate transporter gene. However, they were found to be rather non-specific (see Results).

Instead, the primers designed by the computer for ASC were used for the analysis, and they had the following sequences:

Upper Primer (20-mer): 5' ACTCGGGGCCTCCTCCTGTC 3'

Lower Primer (24-mer) : 5' ATGGCCCAATATAGATGCGAAGAT 3'

The primers for rBAT (*Bertran et al., 1993*) were:

Upper Primer (22mer): 5' TGGCTTCTGTGCTGGTGCTCAT 3'

Lower Primer (24-mer): 5' TTGCGGAAATTTAAATCAGGTTGC 3'

The primers for HPT-1, which was cloned by *Dantzig et al., 1994* were:

Upper Primer (24mer): 5' CAAGCGAAAGTCAGTGAGGATGTA 3'

Lower Primer (24-mer): 5' ACTGCCGAGGGAAAATGTAAAATG 3'

The primers for PepT1 (*Fei et al., 1994*) were:

Upper Primer (22mer): 5' GCTGCCCCCAAGTGACGGAGTT 3'

Lower Primer (19-mer): 5' GGGCGCCAGCGAGGGGTAG 3'

2.12.6 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

cDNA was synthesised from 1 µg DNase-treated total RNA. Before adding the RNA to the reaction mixture, it was denatured by boiling for 5 min then placed on ice for 2 min. The reaction mixture (20 µl final volume) contained 50 pmoles random hexamers (Pharmacia), 5mM MgCl₂, 50mM KCl, 50mM Tris-HCl, pH 8.3, 1mM of each deoxynucleoside triphosphate (dNTP) (Perkin Elmer), 20 U RNasin (ribonuclease inhibitor) (Promega), and 100 U M-MLV reverse transcriptase (BRL Life Technologies, Inc.), and was overlaid with mineral oil. The mixture was incubated for 10 min at 25°C followed by 45 min at 42°C, 5 min at 99°C, then at least 5 min at 4°C. The resultant cDNA was amplified using a DNA thermal cycler (Perkin Elmer Cetus) in a final volume of 50 µl using 2mM MgCl₂, 50mM KCl, 10mM Tris-HCl, pH 8.3, 2.5 U Ampli Taq DNA polymerase (Perkin Elmer), and 15 pmoles of each of the forward and reverse primers, for each 10 µl of cDNA. The reaction mixture was overlaid with mineral oil. After an initial step of 2 min at 95°C, forty PCR cycles were performed, with each cycle comprising 1 min at 95°C and 1 min at 60°C, and then the final step was 60°C for 7 min. The products were examined on a 1.5%, ethidium-bromide-stained agarose gel (0.5 µg EtBr / ml agarose) and their sizes compared to 123-bp or 100-bp molecular weight DNA markers (BRL Life Technologies, Inc.). Glyceraldehyde 3-phosphate dehydrogenase (GAPdH) was used as a positive control, as this is a house-keeping gene known to be expressed constitutively in most cells and in hair follicles (*Little, 1992*). Another positive control used in some experiments was β-glucuronidase, which potentially is involved in extracellular matrix degradation, as it has been shown previously that strong signals are obtained with this in anagen follicles (*Little, personal communication*).

2.12.7 Confirmation of PCR Product Identity

Each product was digested with restriction endonucleases to confirm its identity. To 15µl PCR product was added 4µl 10x Tris Acetate buffer (330mM Tris-acetate pH 7.8, 660mM potassium acetate, 100mM magnesium acetate plus 5mM dithiothreitol), 18.5µl DEPC H₂O and 2.5µl restriction enzyme. The restriction enzymes used were DdeI (10 U/µl, Gibco BRL) and AccI (6 U/µl, Amersham LifeScience). This was incubated at 37°C for about 3 hours, and the products run on a 1% agarose gel to ascertain their sizes. As an additional identity check, the products were gel purified by the QIAEX method (*Vogelstein & Gillespie, 1979*) and subjected to automated fluorescent DNA sequencing, using an Applied Biosystems Automated Sequencer (ABI - Model 373A, version 1.2.0.). The sequences obtained were checked by scanning the Genebank/EMBL database for homology, using the *Align* programme.

2.12.8 QIAEX Method for Gel Purification of DNA

The band to be purified from the agarose gel was cut out from the gel using a scalpel, and placed in an eppendorf tube. Using a QIAEX Gel Extraction Kit, the DNA in the cut piece of gel was extracted by adsorbance to QIAEX silica gel particles, washing with a high salt buffer to remove residual agarose, further washing with an ethanol-containing buffer to remove salts and dyes, and finally eluting the pure DNA from QIAEX into low salt buffer. The method was carried out as follows. 300µl of buffer QX1 and 5µl of QIAEX particles were added to the 100 mg gel slice in an eppendorf tube, and incubated at 50°C for 20-25 minutes, vortexing every 2 minutes. The tube was microcentrifuged for 30 seconds and the pellet washed twice with 500µl of buffer QX2 then twice with buffer QX3. The pellet was then air-dried, and

resuspended in 10 μ l of low salt TE buffer (10mM Tris HCl, 0.1mM EDTA, pH 8). After standing at room temperature for 5 minutes, vortexing every 2 minutes, the sample was microcentrifuged for 30 seconds, and the supernatant, containing the pure DNA, transferred to a clean eppendorf tube.

CHAPTER III

IMPORTANCE OF AMINO ACIDS TO HAIR FOLLICLES. ESPECIALLY ARGININE AND CYSTEINE.

3.1 Introduction

Since hair is comprised almost entirely of protein, amino acids play an important role in hair follicle metabolism and hair formation. The amino acid composition of hair is shown in table 3.1, which is adapted from *Baden et al., 1973*. From table 3.1, it is clear that cyst(e)ine is an important component of hair, so this was subjected to experimental investigation. In addition, arginine was investigated, because like glutamine, arginine is a ω -NH₂-containing amino acid, which could be involved in pH balance (*Kealey, personal communication*), and initial experiments suggested that supplementing William's E medium with extra arginine stimulated hair growth *in vitro* (*unpublished data*).

3.1.1 Development of a Method to Measure the Rate of Soluble Protein Synthesis

The rate of leucine incorporation into follicle proteins should give a measure of the rate of protein synthesis, since leucine makes up approx. 6.1 % of hair protein (table 3.1) and it must be obtained by the follicles from the medium, as it cannot be synthesised by mammalian cells (*Bender, 1985*). Also, as it is one of the more prevalent amino acids in most proteins, it gives higher levels of labelling than the more minor amino acids, so it is good for general labelling of cellular proteins. Therefore, initial attempts were carried out to design an assay to measure leucine uptake in hair follicles and determine its response to changes in nutrient availability.

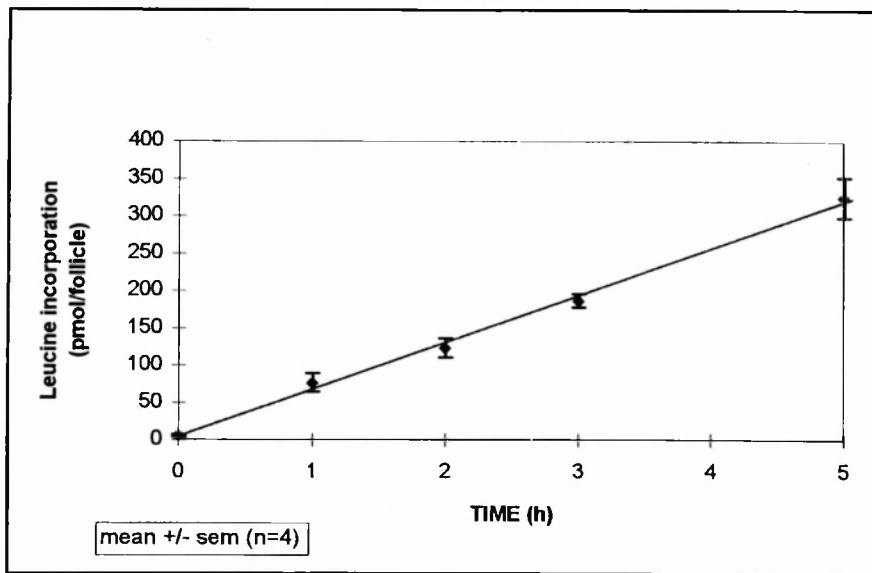
Table 3.1: Amino Acid Composition of Hair (Baden et al., 1973)

Amino acid	% (<i>i.e.</i> residues per 100 residues)
Half-cystine	15.9
Serine	12.2
Glutamic acid	12.2
Proline	8.4
Threonine	7.6
Arginine	6.5
Leucine	6.1
Glycine	5.8
Valine	5.5
Aspartic acid	5.4
Alanine	4.3
Lysine	2.5
Isoleucine	2.3
Tyrosine	2.2
Phenylalanine	1.7
Histidine	0.9
Methionine	0.5

3.1.1.1 Linearity of [U-¹⁴C]Leucine Uptake by Follicles

Follicles which had been cultured for 5 days, growing at the usual rate of 0.34 mm/day in standard Williams' E medium, were used for a protein synthesis assay. Four batches of 5 follicles were removed after 0, 1, 2, 3 or 5 hours of culture with [U-¹⁴C]leucine. The incorporation of [U-¹⁴C]leucine into trichloroacetic acid (TCA)-precipitable protein by the follicles was found to be linear over the 5 hour period under examination (Figure 3.1).

Figure 3.1: Time-course of ^{14}C -Leucine incorporation into soluble protein by isolated human hair follicles



Hair follicles were incubated with ^{14}C -leucine over a time-course of 0 to 5 hours, as described in the text. Results are expressed as the mean \pm SEM for $n=4$ experiments.

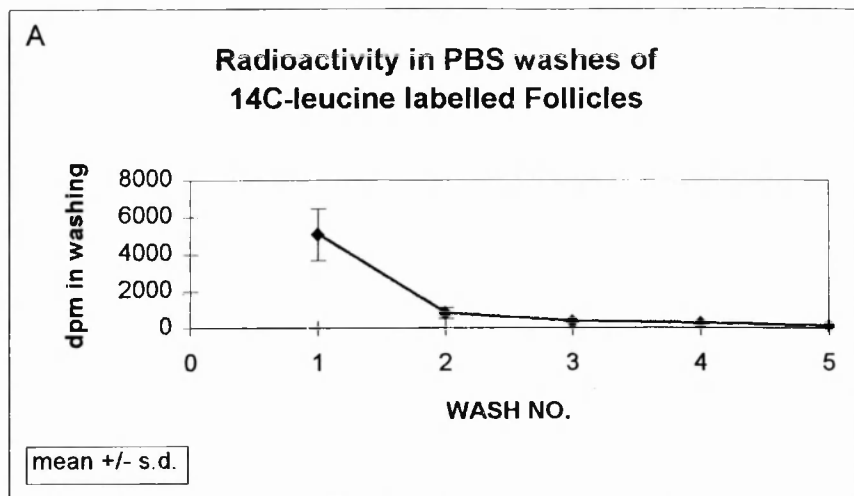
From the results of these experiments, an incubation time of 3 hours was chosen for subsequent protein synthesis experiments, as a convenient time within the linear range.

The radioactivity in the PBS and TCA washes was also counted, to determine the number of washes required for complete removal of non-covalently bound [U- ^{14}C]-leucine. Figure 3.2 shows that three PBS and four TCA washes were found to be sufficient.

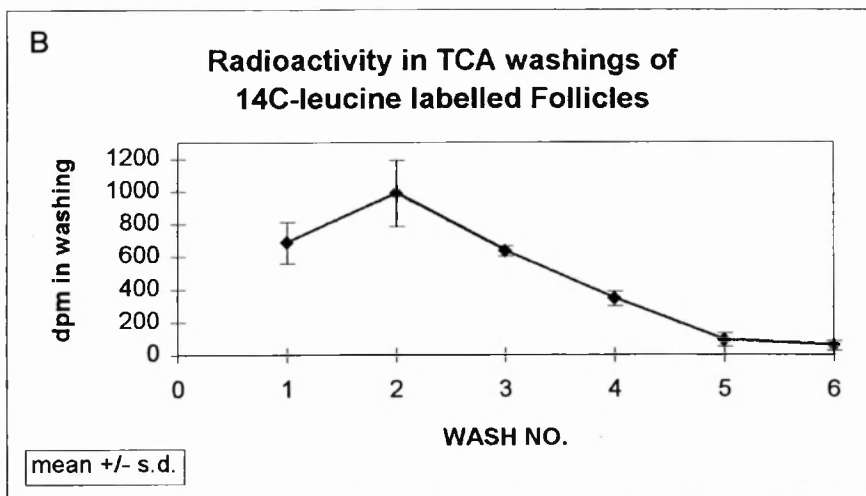
3.1.1.2 Localisation of Leucine Within the Cultured Hair Follicle.

A sample of 5 follicles that had been cultured for 2 days was incubated with [U- ^{14}C]leucine for 3 hours, washed with 4 x 1ml of PBS supplemented with 10mM cold leucine, then fixed in formalin and subjected to histology and autoradiography. Figure 3.3 shows that the [U- ^{14}C]leucine is located mainly in the keratinizing zone and the root sheath in the hair bulb. This confirms that the [U- ^{14}C]leucine, shown previously to be incorporated into protein by hair follicles, is taken up by follicle bulbs. Leucine was not detected in the keratinised hair fibre. Leucine would not be expected to reach the hair shaft in just 3 hours, considering the rate of hair formation. The solubility of the proteins of the hair root differs markedly from those of the hair shaft, since the keratinisation process is still in an early phase in the root. Whereas hair keratin is insoluble in aqueous salt solutions, cells of the keratinizing zone show moderate swelling in distilled water, and are dissociated by homogenisation in EDTA when the hydrogen bonds of prekeratin are opened. Thus, for a labelling time of 3 hours, it is unnecessary to collect insoluble protein. This was confirmed by the low (<15%) percentage of counts found in the insoluble pellet of cell debris following homogenisation.

Figure 3.2

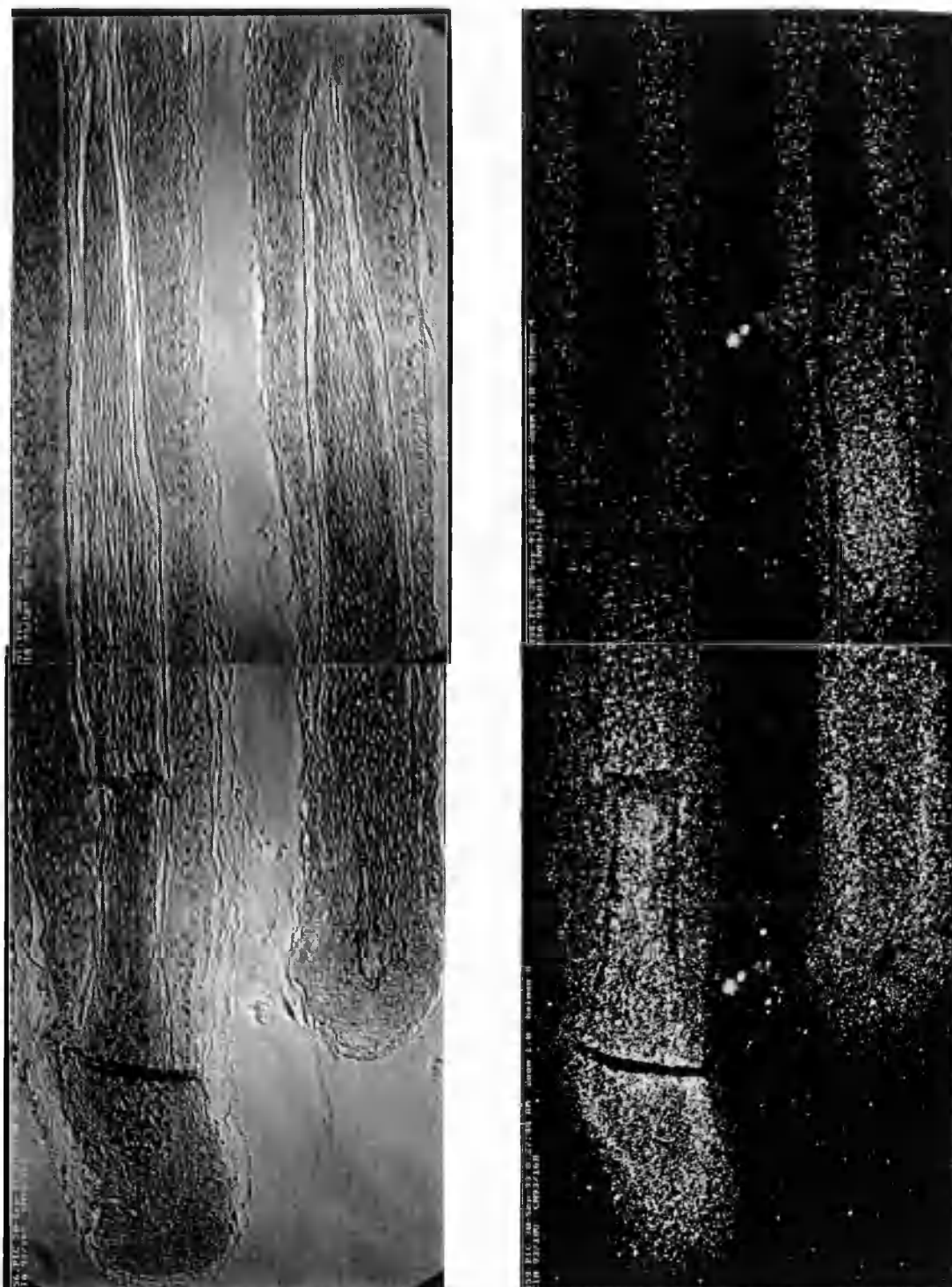


- A. Follicles were incubated with ^{14}C -leucine for 3 hours as described in section 2.5. After removal from the radioactive medium, follicles were washed with successive washes of 1ml PBS supplemented with 10mM cold leucine. The radioactivity in the PBS washes was counted. Results are expressed as mean +/- S.D. for n=3 washes



- B. After washing with PBS/cold leucine, the follicles were placed on filters and washed with successive washes of 5ml ice-cold 5% TCA. The radioactivity in the TCA washes was counted. Results are expressed as mean +/- S.D. for n=3 washes.

Figure 3.3: Autoradiography of [U- 14 C]leucine uptake by isolated hair follicles.



Follicles were incubated with [U- 14 C]leucine for 3 hours were subjected to histology and autoradiography as described in the text. On the left of the figure is the phase-contrast image, and on the right is the image with silver grains showing the location of the radioactivity. Magnification x 120.

3.1.1.3 Effects of Glutamine on Rates of Protein Synthesis by Isolated Hair Follicles

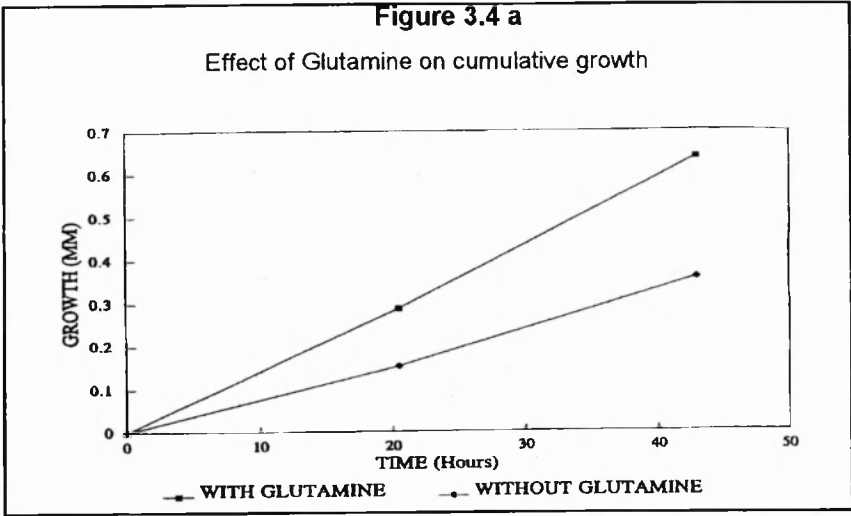
The rate of protein synthesis, as measured by leucine incorporation, was measured in the presence and absence of glutamine, after two days of culture with or without glutamine in the follicle culture medium. As shown in figures 3.4 a & b, use of the protein synthesis assay confirmed that hair growth is retarded in the absence of glutamine. Having shown that glutamine was essential for hair follicle growth and protein synthesis, it was decided to investigate the effects of other amino acids on hair follicle growth and protein synthesis.

3.2 Arginine

Arginine is not classed as one of the essential amino acids, but it may be regarded as such for growth in humans, since it can only be synthesised by the body in limited amounts and a dietary supply is therefore needed (*Thomas & Gillham, 1989*). Possible roles for arginine in the hair follicle may include the following (*Barbul, 1986*):

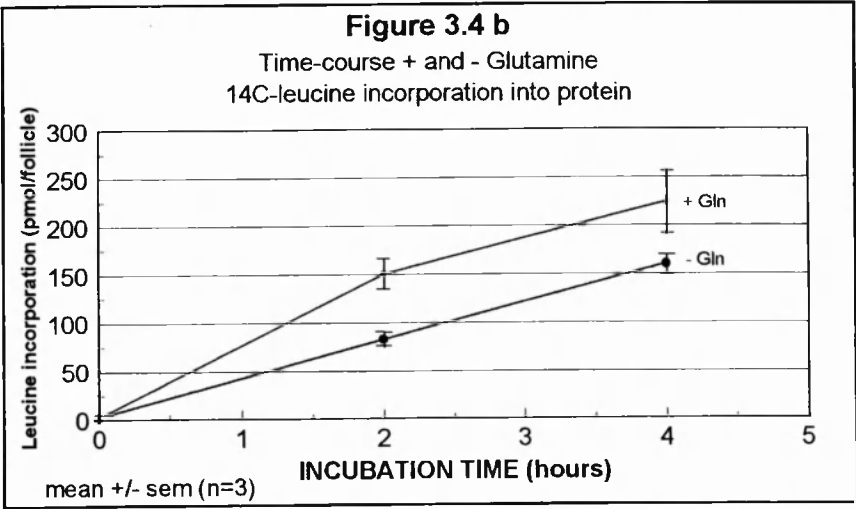
- component of hair follicle structural proteins
- source of metabolic energy
- precursor for polyamines, such as spermidine, which are required for DNA synthesis and cell growth
- urea cycle intermediate, although it is not known whether this pathway occurs completely in hair follicles
- pH regulation, since arginine is a basic amino acid and can be metabolised to provide NH_3 .

Figure 3.4a: Effects of glutamine on cumulative hair follicle growth *in vitro*.



Hair follicles were cultured in the presence or absence of glutamine (2mM) as described in the text. The figure shows the mean cumulative growth of 24 follicles in a representative experiment.

Figure 3.4b:
Effect of glutamine on rate of protein synthesis by isolated hair follicles.



The rate of soluble protein synthesis by hair follicles in the presence or absence of glutamine was measured as the rate of [¹⁴C]leucine incorporation, as described in the text. Results show the mean +/- SEM for n=3 experiments.

3.2.1 Effect of Arginine on Hair Growth

The effect of a range of arginine concentrations on hair growth was measured, by culturing hair follicles in William's E medium containing either 0, 0.287mM, 0.574mM or 1.435mM arginine (*i.e.* 0, 1, 2 and 5 times the normal arginine concentration in William's E medium). As shown in figure 3.5, hair growth does not occur in the absence of arginine, showing that arginine is essential for hair formation. Supplementation of William's E medium with extra arginine above the normal medium level appeared to cause an increase in hair growth, but this was not significant.

3.2.2 Effect of Arginine on the Rate of Soluble Protein Synthesis

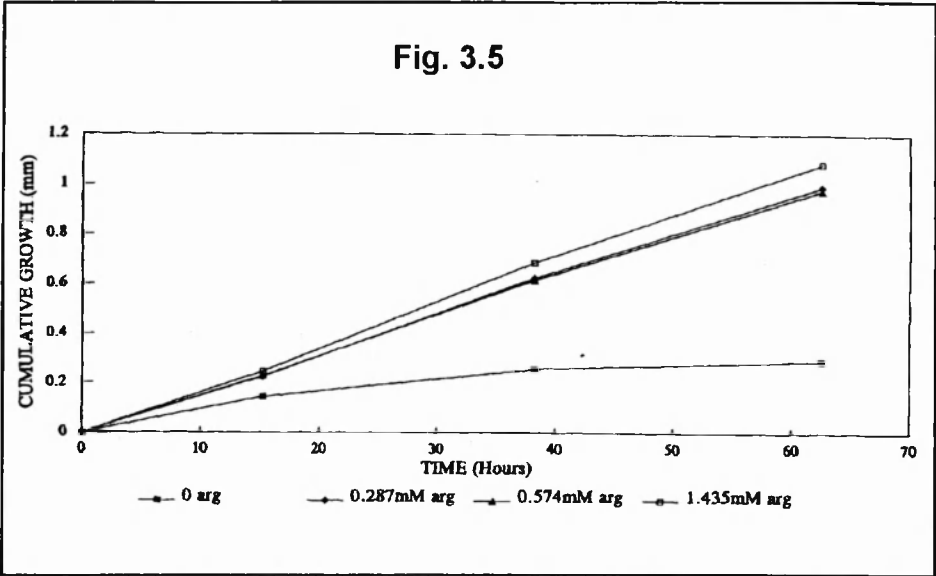
Table 3.3 and figure 3.6 show the mean results from 3 donors. In the absence of arginine, the rate of protein synthesis was very low (approx. 10 pmol/h/follicle). Further supplementation with arginine above 0.287mM had no significant effect, although a trend of increasing rate of protein synthesis with increasing arginine concentration was observed.

Table 3.3 : Effect of arginine on rate of protein synthesis

[Arginine] (mM)	leucine incorporation (pmol/h/follicle) (mean±S.D.)			
	patient 1	patient 2	patient 3	overall
0	11.3 ±3.5 *	11.7 ±1.5 *	13.7 ±3.9 *	12.2 ±3 *
0.287	79.4 ±13.7	46.5 ±8.6	89.8 ± 27.3	71.9 ±25
0.574	92.7 ±10.9	56.0 ±11.8	88.8 ±11.4	79.2 ±20
1.435	110.8 ±6.0 *	55.9 ±7.7	99.9 ±12.5	88.9 ±26

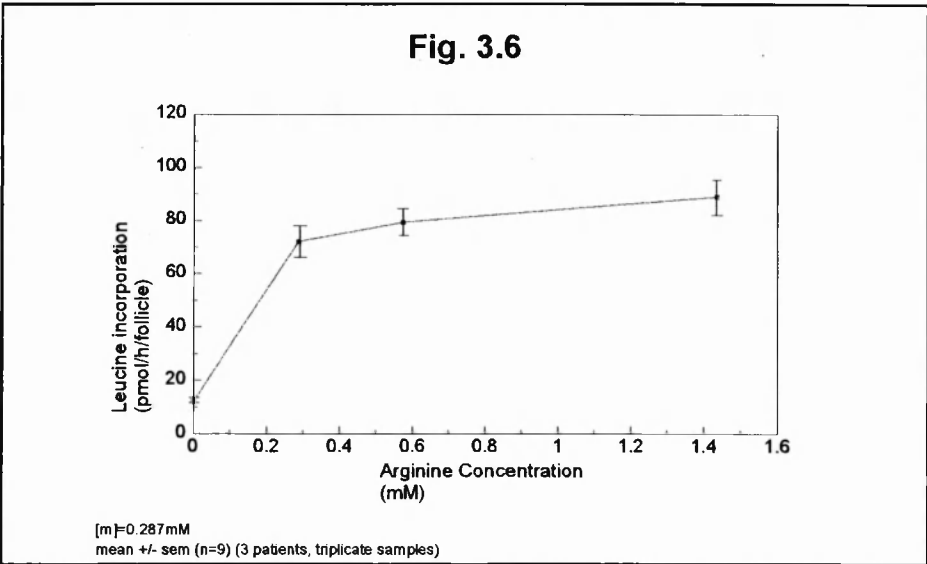
(* = significantly different from 0.287mM level, according to Students t-test, unpaired, $p < 0.05$).

Figure 3.5: Effect of arginine concentration on hair follicle growth *in vitro*



Hair follicles were cultured in medium containing zero, 0.287, 0.574 or 1.435 mM arginine as described in the text. The figure shows the mean cumulative growth of two experiments.

Figure 3.6:
Effect of arginine concentration on rate of protein synthesis by hair follicles



The rate of soluble protein synthesis by hair follicles at zero, 0.287, 0.574 or 1.435mM arginine was measured as the rate of [U-¹⁴C]leucine incorporation, as described in the text. Results show the mean +/- SEM for n=3 experiments.

3.2.3 Location of Arginine Taken Up by Hair Follicles *in vitro*

Uptake of arginine (at $287\mu\text{M}$) was found to be linear for the 5 hours measured, at a rate of approximately $150\text{ pmol/follicle/h}$. Table 3.4 and figure 3.7 show the results from 2 donors.

Time (hours)	Arginine uptake (pmol/follicle)
0	0
1.25	200 ± 29
2.5	405 ± 71
3.5	555 ± 95
5	832 ± 31

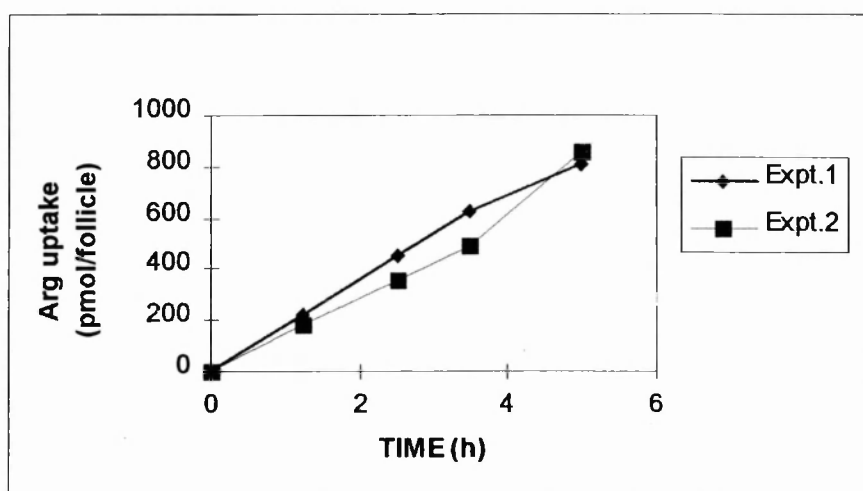
Table 3.4: Uptake of arginine by isolated hair follicles.
(mean \pm range of $n=2$ experiments)

The dissection of hair follicles into separate components and the counting of radioactivity (table 3.5 and figure 3.8) show that about 60% of the arginine is taken up into the root sheaths, 30% into the hair fibre, and only about 10% into the connective tissue sheath (CTS).

Hair part	Arginine taken up in 24h (pmol/follicle) (% in brackets)	
	Patient 1	Patient 2
CTS	96 (6.4)	118 (11.5)
hair sheaths	902 (60.6)	602 (59)
hair fibre	492 (33)	301 (29.5)

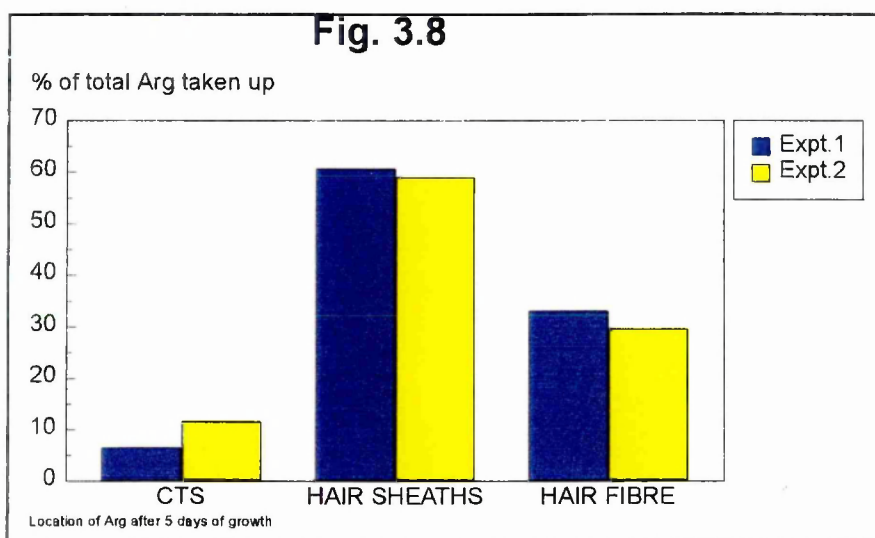
Table 3.5: Location of Arginine taken up by hair follicles after 24h of uptake

Figure 3.7: Time-course of Arginine uptake by isolated hair follicles



Rates of arginine uptake by isolated human hair follicles were measured as described in the text, over a time-course of 5 hours. The figure shows the results of two experiments, with 5 follicles per time-point per experiment.

Figure 3.8: Location of arginine after uptake by isolated hair follicles



Hair follicles were dissected after incubating with ^{14}C -arginine, as described in the text. Results show the percentage of the arginine taken up that was found in the connective tissue sheath (CTS), inner and outer root sheaths or the hair fibre, for two experiments.

The autoradiography revealed that the arginine is located specifically in the inner root sheath (IRS) (figure 3.9).

3.2.4 Metabolism of Arginine to Carbon Dioxide

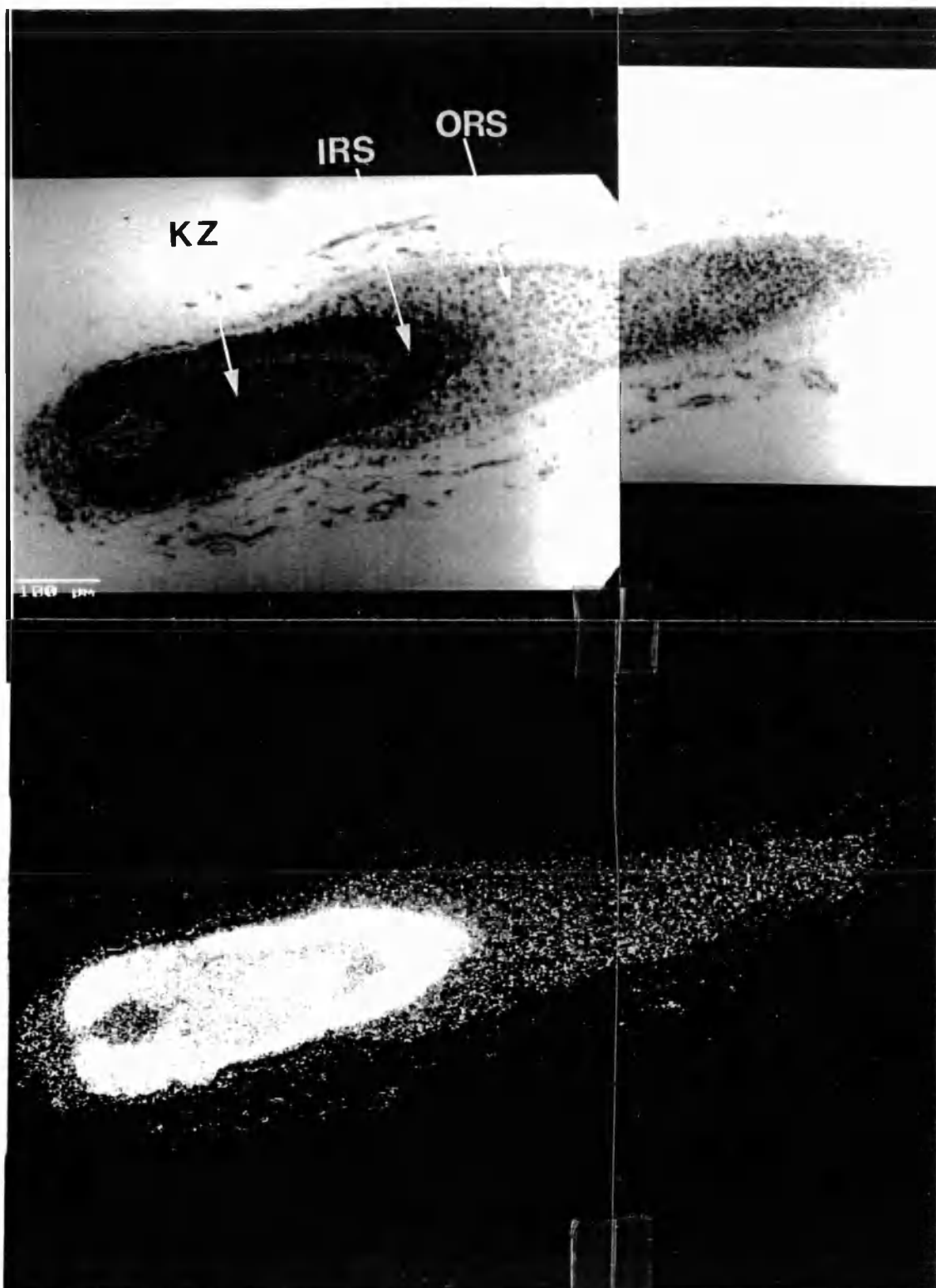
Very small amounts of $^{14}\text{CO}_2$ were produced, approximately 8.9 pmol per follicle in 6 hours, accounting for about 1.1% of the total arginine taken up by hair follicles. The rate of $^{14}\text{CO}_2$ production was found to be linear. The graph shows the mean results of three experiments in which 3 samples of 5 follicles were used per time-point (figure 3.10).

3.2.5 Effect of Arginine Concentration on Lactate Production

The production of lactate during follicle metabolism was shown to be linear for at least 6 hours. Figure 3.11 shows the mean results from 3 patients.

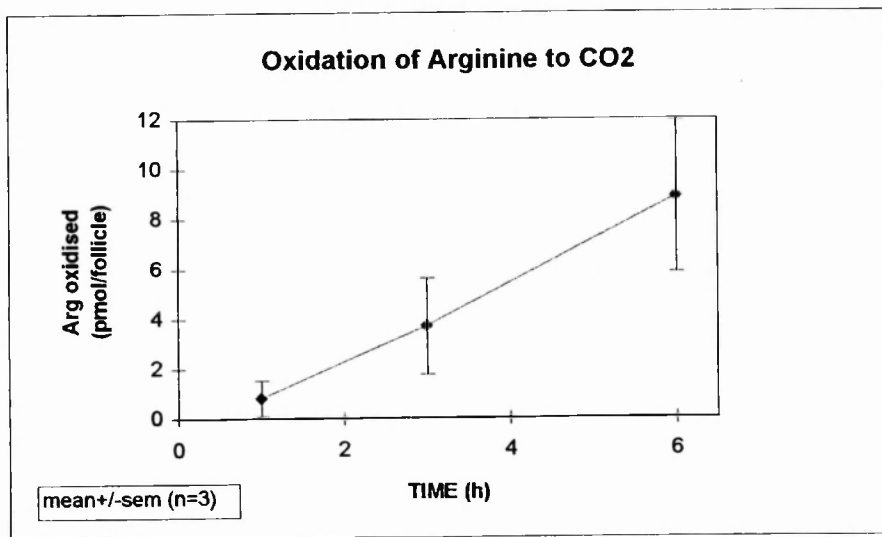
Then, medium containing different arginine levels was assayed for lactate produced by follicles, after 2 days culture. Figure 3.12 shows that at zero arginine, follicles produce significantly less lactate than at the normal Williams' E level of 0.287mM arginine. However, raising the arginine to double its usual concentration had no additional effect. Thus, it appears that 0.287mM arginine is sufficient for normal metabolic production of lactate by follicles, and that some arginine is essential for this process.

Figure 3.9: Autoradiography of a follicle, showing localisation of ^{14}C -arginine primarily in the IRS.



Follicles were incubated with ^{14}C -arginine for 24 hours, then subjected to histology and autoradiography, as described in the text. The figure shows a follicle in longitudinal section. Above phase-contrast, below autoradiography. Magnification x 120.
(IRS = inner root sheath; ORS = outer root sheath; KZ = keratinizing zone)

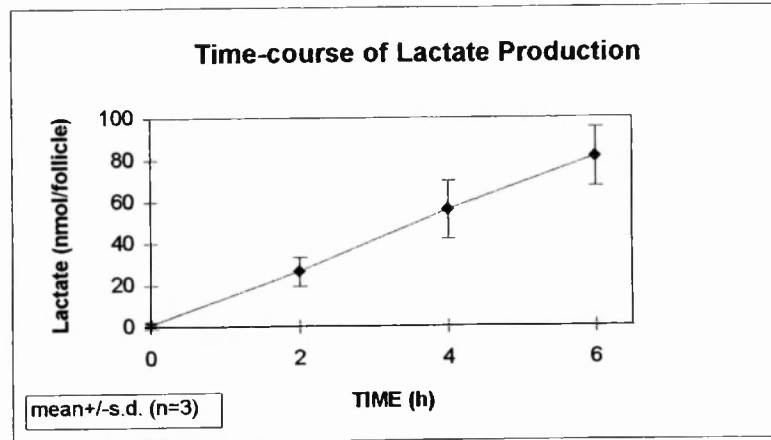
Figure 3.10: Oxidation of Arginine to CO₂ by isolated human hair follicles



Follicles were incubated with ¹⁴C-arginine, and the carbon dioxide produced was collected and counted, as described in the text. The graph shows the mean \pm SEM of 3 experiments in which 3 batches of 5 follicles were used per time-point.

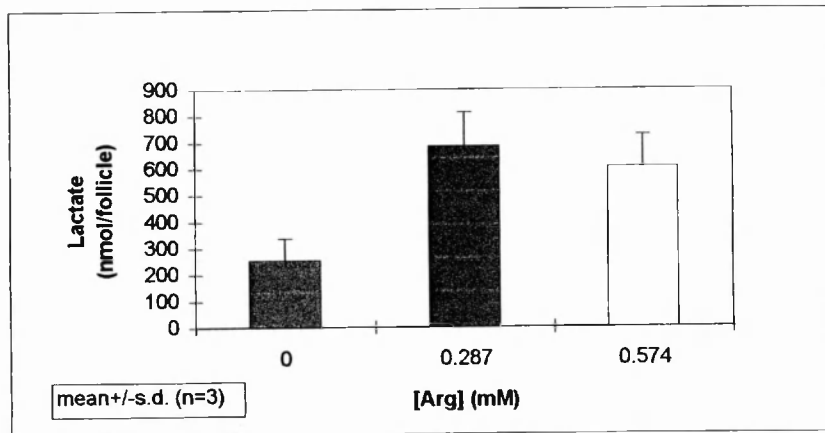
(The 0 time-point did not differ significantly from the 1h time-point)

Figure 3.11: Time-course of lactate production by isolated human hair follicles



Lactate production by follicles was measured over a time course of 0, 2, 4 and 6 hours and found to be linear ($r = 0.9995$) as described in the text. Results show the mean \pm SD for $n=3$ experiments.

Figure 3.12: Effect of arginine concentration on lactate production by isolated hair follicles



Hair follicles were cultured for 2 days in medium containing 0, 0.287 or 0.574 mM arginine, and the medium assayed for lactate as described in the text. Results show the mean \pm SD for $n=3$ experiments.

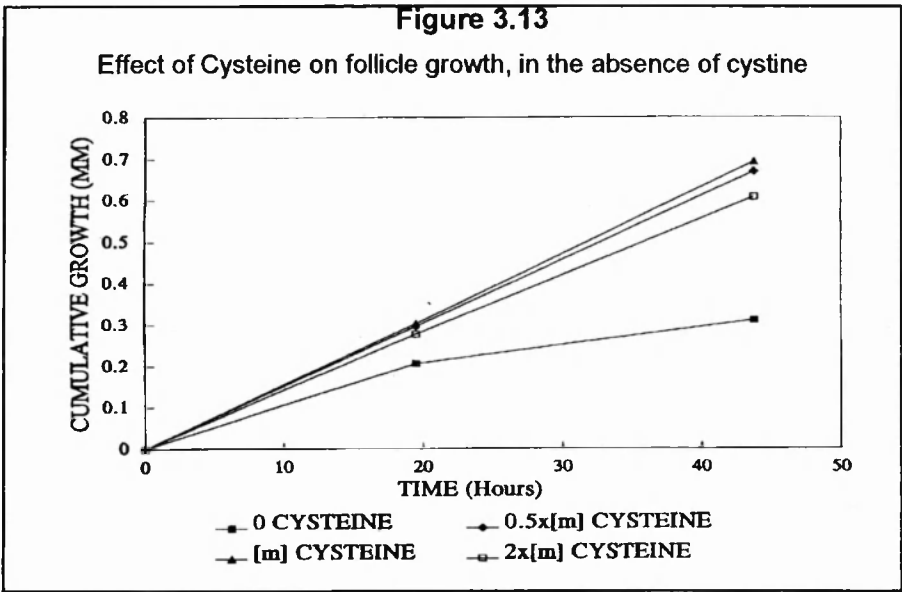
3.3 Cysteine

Table 3.1 shows that cyst(e)ine is an important component of hair. Cysteine, together with the other sulphur-containing amino acid methionine, is known to be important for wool growth in sheep. For example, supplementing the diet of roughage-fed sheep with cysteine or methionine results in an increase in the growth rate of the wool (*Langlands, 1970; Reis, 1967; Reis & Schinckel, 1963*). In contrast, supplementation of the diet of wheat-fed sheep with methionine caused aberrant keratinisation in the follicle, producing weaker, slower-growing hairs (*Dove & Robards, 1974; Reis & Tunks, 1973*). Thus, the level of sulphur-containing amino acids is evidently critical for optimum hair growth and formation. It has been shown in sheep that dietary sulphur is related linearly to the sulphur content of wool proteins (*Broad et al., 1969*). Therefore, the exogenous supply of cyst(e)ine to the follicles will determine the level of these amino acids in hair or wool, and hence the growth and formation of these structures.

3.3.1 Effect of Cysteine on Rate of Soluble Protein Synthesis

Follicles were cultured and assayed in William's E medium 1 containing 0, 0.17, 0.33 or 0.66 mM cysteine with zero cystine, or 0, 0.03, 0.06 or 0.12 mM cystine with zero cysteine. These concentrations were chosen to give a range covering 0 to 2 times the normal Williams' E concentration (which is 0.33mM cysteine plus 0.08mM cystine). In the absence of both cysteine and cystine, the follicles grew very poorly, while the other cys concentrations tested all gave rise to normal *in vivo* growth rates (approx. 0.35 mm/day). The growth curves are shown in figure 3.13. A similar pattern was seen in the rates of protein synthesis, *i.e.* the rate of protein synthesis in the

Figure 3.13: Effect of Cyst(e)ine on hair follicle growth *in vitro*



Hair follicles were cultured in medium containing 0, 0.5x[m], [m] or 2x[m] cysteine, in the absence of cystine, as described in the text. (Where [m] = normal concentration of cysteine in William's E medium, 0.33 mM). Results show the mean of 24 follicles per cysteine concentration.

The growth of follicles in the absence of cystine, with cystine at 0, 0.5x[m], [m] or 2x[m] (where [m] = 0.08 mM cystine) was similar (data not shown).

absence of both cysteine and cystine was very low (less than 20% of the control rate) while the cysteine concentrations above 0.17mM and the cystine concentrations above 0.03mM did not differ significantly from the control rate (Tables 3.6 a&b and Figure 3.14). These results confirm that cysteine/cystine is essential for hair protein synthesis but suggest that the cyst(e)ine concentration in William's E is at least double that necessary to support normal protein synthesis.

[Cystine] (mM)	[U- ¹⁴ C] Leucine incorporation (pmol/h/follicle) (mean±S.D.)
0	14.3 ±3.5 *
0.03	65.6 ±13.1
0.06	85.6 ±3.6
0.12	85.4 ±8.1

Table 3.6 a: Effect of cystine in the absence of cysteine.
(* = significantly different from 0.03mM level, according to Students t-test, $p < 0.05$).

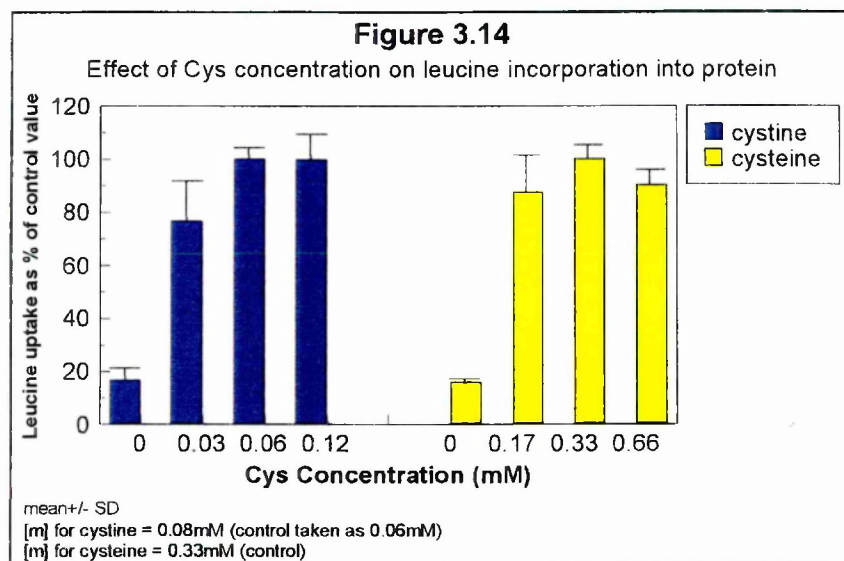
[Cysteine] (mM)	[U- ¹⁴ C] Leucine incorporation (pmol/h/follicle) (mean±S.D.)
0	16.8 ±1.2 *
0.17	91.9 ±14.1
0.33	105 ±4.2
0.66	94.5 ±5.9

Table 3.6 b: Effect of cysteine in the absence of cystine.
(* = significantly different from 0.33mM level, according to Students t-test, $p < 0.05$).

3.3.2 Effect of Decreasing Cysteine on Hair Growth

The level of cyst(e)ine in William's E medium (0.33 mM cysteine + 0.08 mM cystine, or 77.3mg/l cysteine equivalents) is considerably higher than in plasma

Figure 3.14: Effect of Cyst(e)ine concentration on rate of protein synthesis by hair follicles



The rate of soluble protein synthesis by hair follicles at various concentrations of cysteine or cystine was measured as the rate of $[U-^{14}C]$ leucine incorporation, as described in the text. Results show the mean \pm SD for $n=3$ experiments. The normal concentration of cysteine in William's E medium is 0.33 mM, and of cystine it is 0.08 mM.

(approx. 100 μ M (or 15mg/l) of cysteine equivalents, *Geigy Scientific Tables 3, 1984*). In addition, the protein assay indicated that William's E medium contains more cyst(e)ine than is required by hair follicles. Therefore, it was decided to look at lower, more physiological cysteine concentrations, in order to identify the minimum level required to maintain hair follicle viability. The follicles continued to grow, albeit at reduced rates, even at 1/55 of the normal William's E medium cyst(e)ine concentration, which is approximately one tenth of the plasma concentration, (figure 3.15).

3.3.3 Effect of Decreasing Cysteine on Hair Fibre Diameter

Diameter is an important hair property which strongly influences bulk hair characteristics because the softness, manageability and strength of hair are related to fibre diameter (*Yin et al, 1977*):

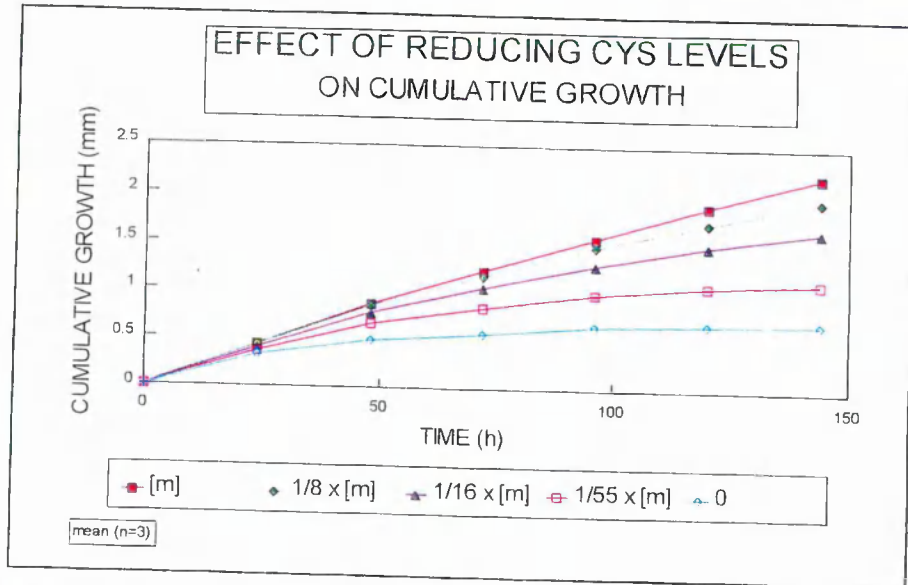
$$\text{Force required to bend fibre (F)} \propto d^4$$

Not only was the growth rate reduced at lower cyst(e)ine concentrations, but the hair fibre produced appeared to have a markedly reduced diameter (figure 3.16).

3.3.4 Effect of Reversing the Decreased Supply of Cysteine

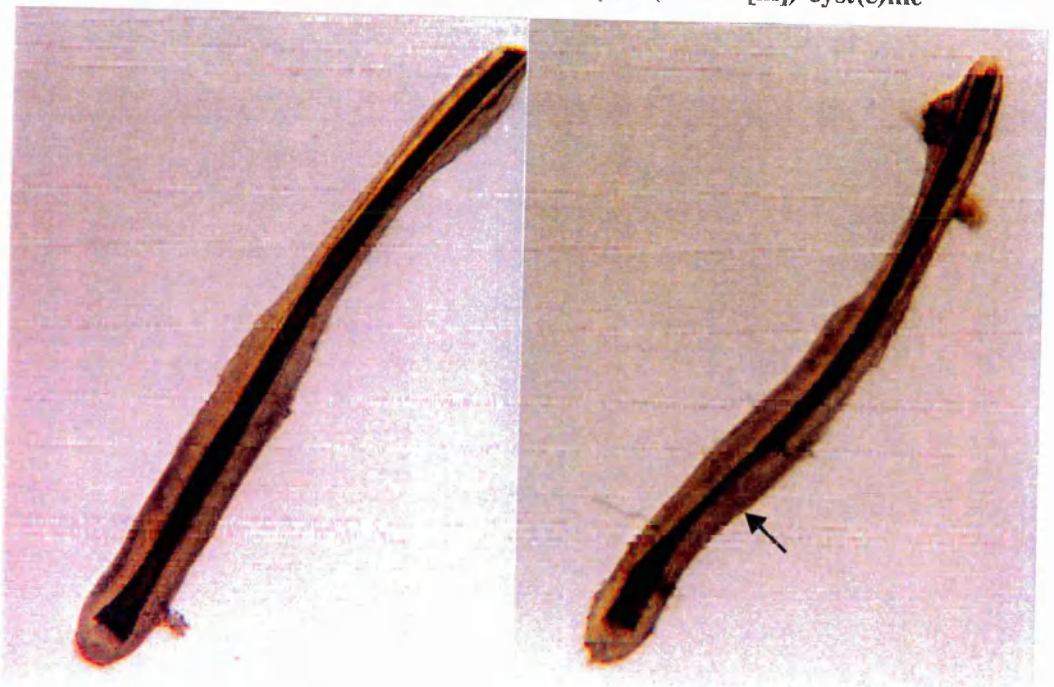
The fibre thinning and growth retardation caused by low cyst(e)ine levels were reversed successfully by restoring the follicles to full Williams' E levels of cyst(e)ine after 2 days of culture in low (1/55 x the concentration in normal William's E) cyst(e)ine medium. The combined results of these experiments are shown in figure 3.17, and the appearance of a typical follicle after such an experiment is illustrated in figure 3.18.

Figure 3.15: Effect of cyst(e)ine concentration on hair follicle growth *in vitro*



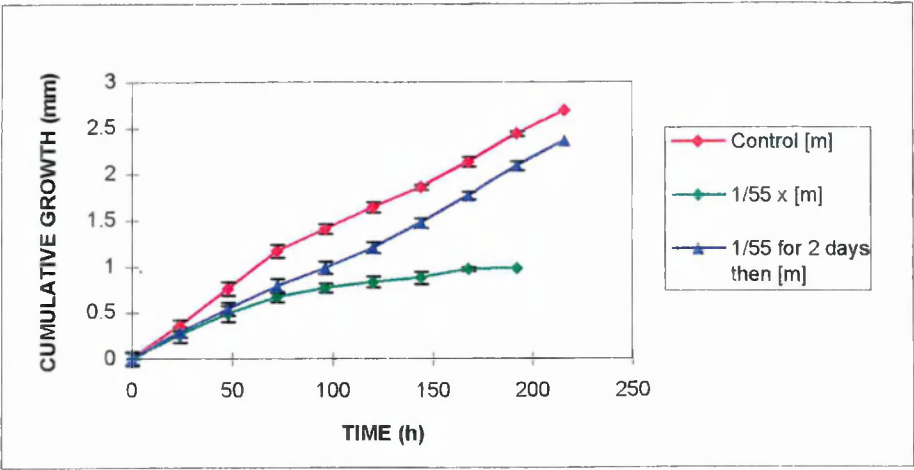
Hair follicles were cultured in medium containing 0, 1/55x[m], 1/16x[m], 1/8x[m] or [m] cysteine and cyatine, where [m] = normal medium concentration of cyst(e)ine (0.33 mM cysteine plus 0.08 mM cystine), as described in the text. Results show the mean of n=3 experiments.

Figure 3.16: Photograph of follicle grown at 9 μ M (1/55 x [m]) cyst(e)ine



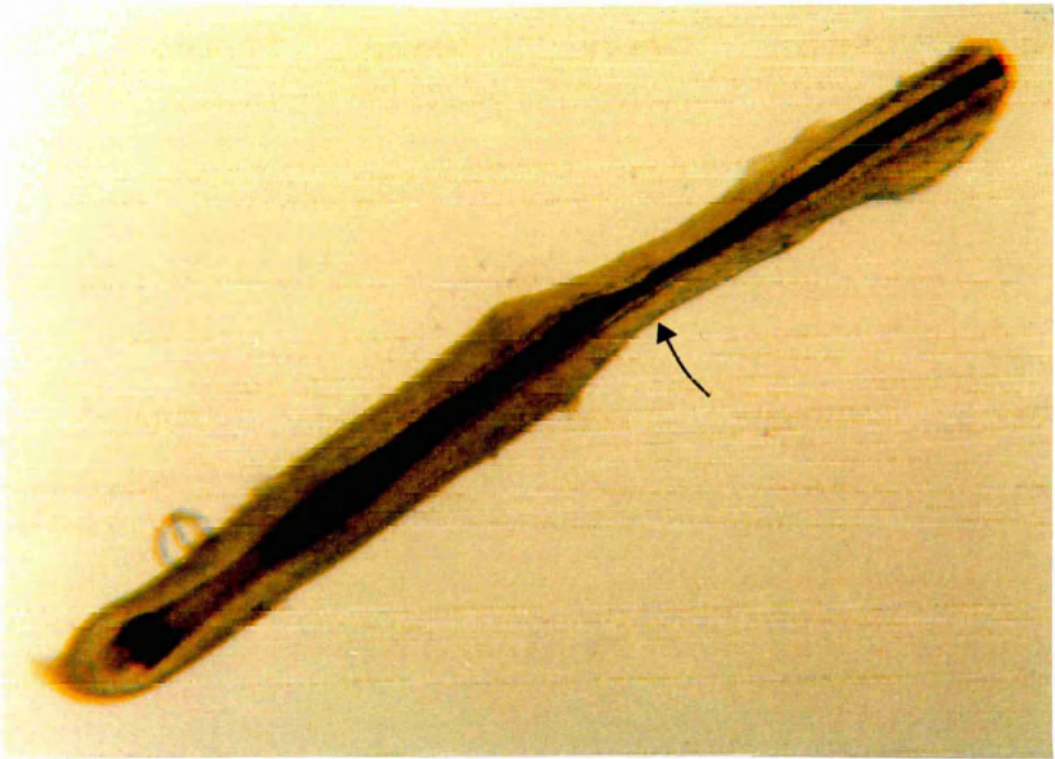
On the left is a control follicle, grown at [m] cyst(e)ine. On the right is a follicle grown at 1/55x[m] cyst(e)ine. Note reduction in hair fibre diameter at arrow.

Figure 3.17: Effects of reversing the decreased supply of cyst(e)ine on hair follicle growth



Hair follicles were grown at control ($[m]=0.49\text{mM}$ half-cystine equivalents), reduced (0.009 mM half-cystine equivalents) or reversed (0.009 mM for 2 days then control) cyst(e)ine levels, as described in the text. Results show the mean \pm SD of $n=3$ experiments.

Figure 3.18: Photograph of follicle which has been grown at $9\mu\text{M}$ ($1/55x[m]$) cyst(e)ine for 2 days and then at control ($[m]$) cyst(e)ine levels.



Note reduction in hair fibre diameter at arrow.

3.4 Discussion

This chapter demonstrated that arginine is essential for hair follicle growth *in vitro*. Although arginine is not normally listed as one of the "essential amino acids", it is normally synthesised in the liver, so for a hair follicle growing in isolation, it is perhaps not surprising that a supply of arginine is required. Arginine is an important component of histones, needed for chromatin formation, and thus it is important for rapidly dividing cells which are synthesising DNA, such as the cells of the hair follicle.

The conversion of arginine into carbon dioxide, the ultimate product of oxidative pathways, was found to occur in hair follicles under normal culture conditions. This shows that arginine can be metabolised to provide energy, probably via entry into the tricarboxylic acid cycle. The rate of $^{14}\text{CO}_2$ production was found to be linear. However, only very small amounts of $^{14}\text{CO}_2$ were produced, approximately 1.7 pmol per follicle per hour, accounting for only 1.1% of the total arginine taken up by follicles (see section 3.2.3). This is equivalent to one tenth of the amount of arginine that is incorporated into hair fibre. Therefore, this is evidently not a major route for arginine utilisation in follicles. As a comparison, glutamate has been shown to be metabolised to CO_2 at a rate of 34.4 ± 1.6 pmol/follicle/h (mean \pm sem) (Wiechers JW, *personal communication*), which is equivalent to 37 times the amount of glutamate that is incorporated into hair fibre (Matheson H, *personal communication*). Both glutamine and arginine can be converted into glutamate as a route of entry into the TCA cycle as α -ketoglutarate. It is possible that if the preferred substrate, glutamine, were unavailable then more arginine may become diverted into energy producing metabolism. However, the analogous situation of reducing the glutamine concentration does not influence the utilisation rate of externally supplied glutamate as an energy source (Wiechers JW,

personal communication). Preliminary results with arginine (not reported) are inconclusive, and further investigation is required.

Lactate production is a measure of the main energy metabolism of follicles, aerobic glycolysis. It was found that at zero arginine, follicles produce significantly less lactate than at the normal Williams' E level of 0.287mM arginine, over a period of 2 days. However, raising the arginine to double its usual concentration had no effect. Thus, it appears that 0.287mM arginine is sufficient for normal metabolic production of lactate by follicles, but that some arginine is essential. It is likely that the follicles without arginine die within 2 days, so the reduction in lactate production under these conditions is not surprising. However, this study shows that the stimulation of hair growth brought about by supplementation with arginine does not appear to be a result of arginine stimulating energy production by aerobic glycolysis (although it should be noted that 1mM arginine (*Granger, personal communication*) was required for growth stimulation, *i.e.* more than the double medium concentration, 0.574mM, tested here).

Perhaps a more likely role for arginine in the hair follicle is a structural one, since it is an abundant amino acid in hair and follicle proteins. Hair protein is known to consist of approximately 6.5% arginine (*Baden et al., 1973*). The fate of arginine taken up into follicles was examined to see whether it ends up in specific localisations or structures. Arginine uptake into whole follicles was found to be linear for the 5 hours measured, at a rate of approximately 150 pmol/follicle/h in Williams' E medium, which contains 287 μ M arginine. This compares with a rate of uptake of leucine of about 260 pmol/follicle/h in medium containing 572 μ M leucine (*Riches C, unpublished*). If we assume that uptake is linearly related to concentration, 287 μ M leucine would give an uptake rate of 130 pmol leucine/follicle/h, or 572 μ M arginine

would give an uptake of 299 pmol arginine/follicle/h. *i.e.* the uptake rate of arginine is similar to that of leucine, at equivalent concentrations, with this assumption. Leucine is an amino acid which is important for protein synthesis, and constitutes approximately 6.1 % of hair protein (*Baden et al., 1973*). This similarity of uptake may therefore lend support to the hypothesis that the primary role of arginine in hair follicles is as a protein component. However, no experimental data are available to substantiate the assumption of a linear relationship between uptake and concentration, so this is only supposition. In contrast, a comparison of arginine uptake with that of glutamate shows a large difference. Data for whole follicle uptake is not yet available for glutamate, but uptake into the hair fibre has been measured under similar conditions for glutamate as was done for arginine in this study (*Matheson H, personal communication*). Arginine uptake at 287 μ M arginine is 17.8 pmol / h / hair fibre (measured over 20-24h, therefore assuming linearity with time), whereas glutamate uptake at 340 μ M glutamate is only 0.9 pmol/h/hair fibre (measured over 5h). This almost 20-fold difference is remarkable, especially since the concentration of arginine is actually lower than that of glutamate (both experiments were carried out in full William's E medium), so the opposite trend might have been expected if purely considering concentration effects. One implication of this result could be that arginine is more important than glutamate to hair follicles. Alternatively, perhaps the cellular uptake mechanism for glutamate is limiting, and chapter V assesses this possibility. Of course, it is also possible that glutamate is equally important to follicles, but that its role is different. Indeed, as has already been discussed, it is more important than arginine as a metabolic fuel, and whole follicle glutamate uptake measurements should be performed in order for a complete comparison to be made.

Not only is the arginine actively taken up, but it is subsequently localised very specifically to particular structures within the follicle. About 60% of the arginine is taken up into the root sheaths, in particular the inner root sheath (IRS), 30% into the hair fibre, perhaps specifically in the medulla, and only about 10% into the CTS. Since the IRS contains trichohyalin, a protein rich in arginine, this result is consistent with current knowledge about hair follicle structure and formation. Trichohyalin is an intermediate filament-associated protein that is believed to associate with keratin intermediate filaments of the IRS and medullary cells of the developing hair follicle (*Rothnagel & Rogers, 1986*). The exact role and function of trichohyalin is not yet known, but it has been postulated that it may serve as a cross-linking molecule, conferring a strong yet flexible scaffold-like structure to the tissue (*Lee et al., 1993*). Thus, trichohyalin may be of major importance in conferring structural function to the IRS, which will in turn determine the morphology of the hair fibre produced by the follicle. Trichohyalin consists of 30% arginine, and in addition many arginine molecules are converted to citrulline by the enzyme peptidylarginine deiminase (*Rogers & Rothnagel, 1983*). It is therefore plausible that a significant role for arginine in the hair follicle is to form trichohyalin. The specificity of the arginine location suggests the existence of specific transport processes to concentrate the arginine in these cell layers. The cellular transport mechanisms for arginine, discussed in section 6.4, may provide a key to increasing the supply of arginine to the part of the follicle where it is needed most.

As well as being incorporated directly into proteins itself, it is possible that arginine stimulates the rate of incorporation of other amino acids into protein, to give an overall increase in the rate of hair follicle protein synthesis. This was measured by

looking at the incorporation of leucine into soluble protein. Arginine was found to be essential for normal rates of protein synthesis by follicles. This is as expected, since a major product of arginine catabolism is ornithine, which is essential for polyamine synthesis and cell cycle progression (*Pegg & McCann, 1982*). Further supplementation with arginine above 0.287mM had no significant effect, although there was a trend of increasing rate of protein synthesis with increasing arginine concentration. Although the increase in protein synthetic rate from 0.287 to 1.435 mM arginine is small, it could still account for the increased (4.7%) growth rate observed when follicles are supplemented with 1mM arginine *in vitro* (*Granger, personal communication*) after 7 days treatment. Since the physiological (blood) level of arginine is approximately 60-80 μ M (*Hagenfeldt & Arvidsson, 1980*), this may well give a protein synthetic rate that is significantly lower than the rate at the medium concentration of arginine, so this should be tested. Thus, it is possible that there is a small effect of arginine acting to stimulate follicle protein synthesis, but this seems unlikely to represent the primary mechanism by which arginine stimulates hair growth. It would be useful to measure the incorporation of arginine itself into protein at raised arginine concentrations, to ascertain whether this is increased.

In this chapter, the role of cysteine in hair follicles was also studied . The presence of cysteine was shown to be essential for hair growth and soluble protein synthesis *in vitro*. Cysteine is a major component of hair proteins (15.9 % half-cystine), so this is not surprising (*Baden et al., 1973*). Since doubling or halving the normal concentration of cyst(e)ine in William's E medium had no significant effect on the rate of protein synthesis, it was concluded that the amount of cyst(e)ine present in William's

E medium (0.33 mM cysteine + 0.06 mM cystine) is more than the follicle requires for normal biosynthetic processes. Therefore, the level of cyst(e)ine in the medium was reduced further to find the minimum level required to sustain hair growth. An interesting phenomenon observed was that not only did linear hair fibre growth decrease at low cysteine levels, but the fibre diameter also decreased. This effect was reversible, enabling a hair fibre to be grown with a thin region along its length. It would appear, therefore, that the cysteine supply regulates the diameter of the hair fibre being formed. In support of this, a similar effect has been seen in sheep, where infusion of cysteine for 21 days at approximately 4 g cysteine per day led to a 1.3-2.9 μm increase in wool fibre diameter (*Fratini et al., 1994*). Interestingly, it has been observed that the hair normally produced *in vitro* widens during culture (personal observation). One suggestion is that this is due to swelling by water, as the fibres are submerged in liquid, but these observations with cysteine suggest that the widening could be caused by the high cysteine present in normal William's E. The concentration of the other sulphur-containing amino acid, methionine, does not affect the hair fibre diameter, but if the methionine level in the medium is reduced, hair follicle growth merely slows down and eventually stops (*Parmar P., personal communication*). This may be explained by the importance of methionine for polyamine synthesis via S-adenosyl methionine (SAM). In sheep, methionine can replace cysteine, but cysteine cannot replace methionine. Polyamines are essential for wool growth (*Reis, 1988*), and the same probably applies to hair (*Hynd & Nancarrow, 1996*).

The thin fibre produced at low cysteine may provide a model for the regulation of hair diameter, for example by seeing whether the diameter can be increased again using agents other than cysteine, *eg.* growth factors. A possible candidate is insulin-like

growth factor I (IGF-1), which is believed to stimulate cysteine uptake into sheep skin (Harris *et al.*, 1993) and may therefore be able to maximise the utilisation of the small amount of cysteine available. IGF-1 inhibits apoptosis in fibroblasts (Harrington *et al.*, 1994), and it (or insulin) is needed to maintain follicles *in vitro*. Perhaps lack of cysteine results in apoptosis? However, no degeneration was observed in the follicles at low cysteine levels, so this seems unlikely. It is possible that the effect of cysteine on diameter is a genetic effect, the cysteine concentration affecting the regulation of gene expression. It would be interesting to investigate whether genes in human hair follicles are switched on or off at low cysteine levels, as it has been observed recently in sheep wool follicles that an increase in dietary cysteine causes a 5-6 fold increase in the expression of a gene family encoding cysteine-rich wool cortex proteins (Fratini *et al.*, 1994). Since regulation of keratin gene expression is known to be important in hair follicle differentiation (Powell *et al.*, 1991), the level of cysteine may affect the overall differentiation process. Histology and electron microscopy are being carried out on the hair follicles from the experiments in this thesis, and so far appear to show a compensatory increase in the thickness of the outer root sheath at the region of fibre thinning, possibly with abnormal intracellular spaces.

CHAPTER IV

MOLECULAR BIOLOGY OF AMINO ACID TRANSPORTERS

4.1 Introduction

As discussed in chapter III, amino acids are important nutrients for hair follicles. So far the effect on hair follicles of altering the concentration of these substrates available to them has been considered, but it is also important to know what is happening at the cellular level in terms of uptake of the amino acids. It may be that the cellular transport of a particular amino acid is limiting to the follicular needs for that amino acid. For example, it is known that in hepatocytes, system A-mediated transport is the rate-limiting step in alanine catabolism (*Handlogten & Kilberg, 1983*).

As a first step to understanding the transport processes which occur in human hair follicles, the polymerase chain reaction (PCR) was used to identify which transporters are expressed in follicles. In addition, outer root sheath (ORS) cells were probed, as measurement of amino acid transport is more easily carried out on cells than on whole follicles, and ORS cells were chosen as they are the first cell layer likely to be encountered by amino acids coming from the blood. As ORS cells are relatively slow-growing, the rapidly-proliferating LSDM3 cell-line was chosen for carrying out initial experiments, so these cells were also subjected to PCR, to check that they are similar in their transporter profile to follicles, and are therefore a suitable model.

4.2 Cell Culture

Figure 4.1 shows a typical ORS cell culture. The cells explanted from the follicle pieces and formed characteristic cobblestone arrangements of keratinocytes.

Figure 4.1: A typical Outer Root Sheath Cell Culture

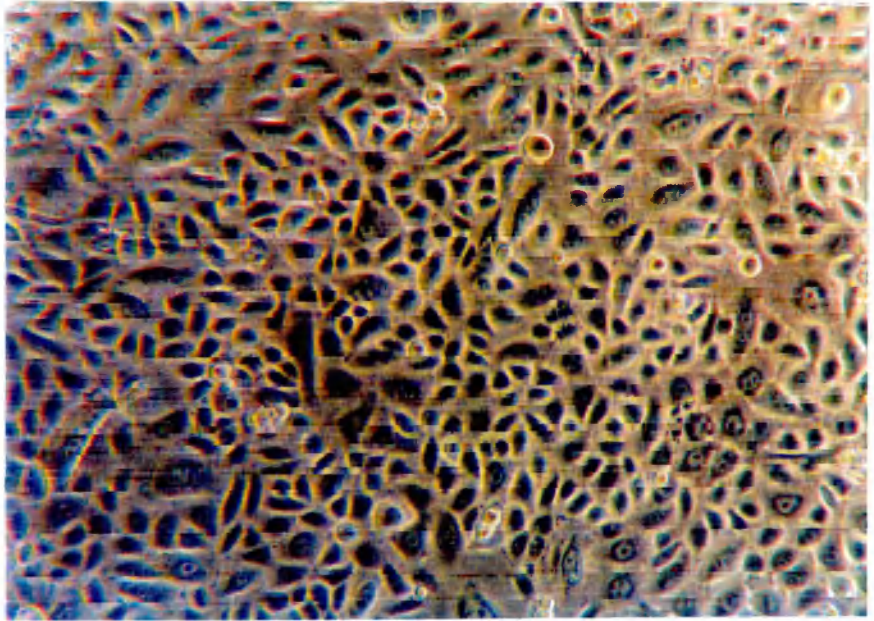
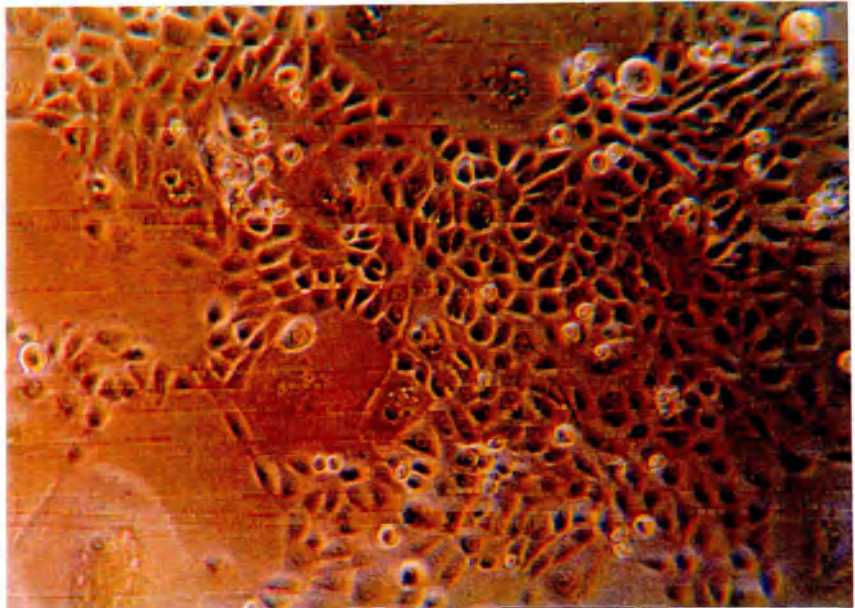


Figure 4.2: LSDM3 Cells in Culture



The ORS cultures used were as follows:

ORS18 was derived from a 60 year-old female. The follicles from the same patient were also homogenised and used as an RNA source, so that ORS cells and follicles from the same donor could be compared. The ORS18 cells were harvested at passage number 3. ORS22 was derived from a 47 year-old female. ORS23 was derived from a 47 year-old female. Both ORS22 and ORS23 were harvested at passage no.2.

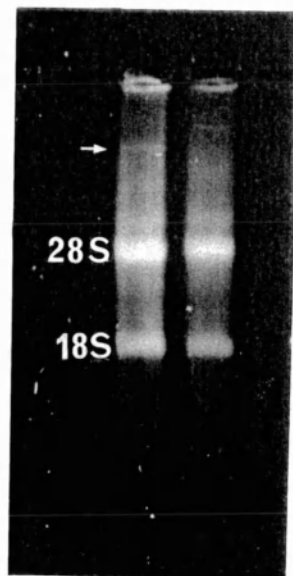
Figure 4.2 shows LSDM3 cells in culture. They have a more flattened appearance, but still form cobblestone arrangements of cells. Two lots of LSDM3 cells were harvested, at passage numbers 39 and 41.

4.3 RNA Preparation

The RNA extracted from ORS18 and run out on a 1% agarose gel is shown in figure 4.3. The characteristic bands can be seen (see Chapter II), confirming that the RNA is not degraded. After DNase treatment, some of the additional bands have disappeared (*e.g.* arrow in figure), suggesting that these were contaminating genomic DNA, and are removed by DNase treatment.

The importance of DNase treatment was seen when primers of rather low specificity were used to probe for the System ASC gene in follicle cDNA. Several bands were obtained initially with cDNA reverse-transcribed from untreated RNA, but after DNase treatment of the remaining RNA prior to cDNA synthesis, specific bands were obtained for ASC (figure 4.4). Presumably, the extra bands initially obtained were a result of the primers binding to genomic DNA.

Figure 4.3: RNA extracted from ORS cells, before and after DNase treatment

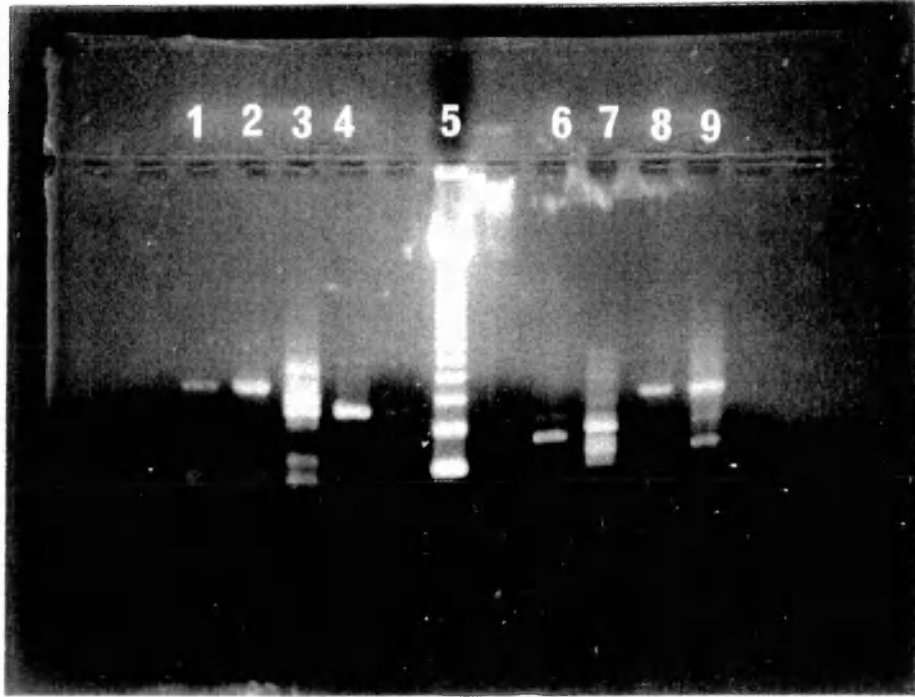


1st lane = before DNase treatment

2nd lane = after DNase treatment

RNA was extracted from ORS cells and treated with DNase, as described in the text. The figure shows the RNA before (lane 1) and after (lane 2) DNase treatment. The characteristic bands can be seen (*e.g.* 28S and 18S rRNA), confirming that the RNA is not degraded. After DNase treatment, some of the additional bands have disappeared (*e.g.* see arrow), suggesting that these were contaminating genomic DNA, removed by DNase treatment.

Figure 4.4: PCR products for transporter system ASC, using low-specificity primers, to show the importance of DNase treatment of RNA prior to cDNA synthesis for PCR



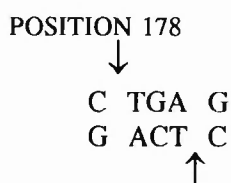
PCR was carried out on cDNA made from either DNase-treated or untreated RNA, using primers specific for GAPdH and β -glucuronidase and two pairs of primers with low specificity for the ASC gene, ASC 1+2 and ASC 2+3. The low specificity primers gave rise to several bands on untreated RNA samples, but a single band was obtained with DNase-treated samples. See text for details.

- lane 1 = GAPdH(DNase-treated)
- lane 2 = GAPdH(untreated)
- lane 3 = ASC(untreated)
- lane 4 = ASC 1+2(DNase-treated)
- lane 5 = 123bp DNA ladder
- lane 6 = ASC 2+3(DNase-treated)
- lane 7 = ASC 2+3(untreated)
- lane 8 = β -glucuronidase(DNase-treated)
- lane 9 = β -glucuronidase(untreated)

The binding of the primers to genomic DNA was also demonstrated by a control PCR reaction in which untreated RNA was put into the PCR reaction instead of cDNA. No bands would be expected, since taq DNA polymerase does not bind to RNA, but as figure 4.5 shows, several bands were actually obtained, so these must have come from genomic DNA. These primers were superseded by more specific ones for subsequent experiments, but DNase treatment was carried out as a precaution on all RNA samples.

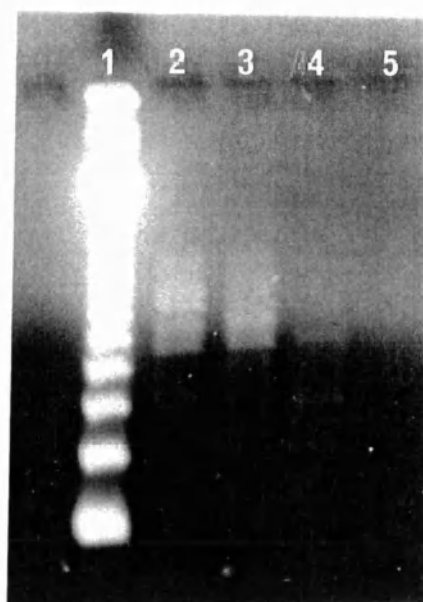
4.4 PCR of System ASC

As shown in figure 4.6, follicles (F), ORS cells (O) and LSDM3 cells (L) were all found to express the SATT gene which encodes transport system ASC. The sequence of the expected product has sites for the restriction enzyme DdeI, which would digest the 446bp product into two fragments of lengths 178 and 278 bp.



Digestion did indeed give rise to fragments of the expected size, as shown in figure 4.7. Since this analysis is based purely on fragment size and number, it is not direct proof that the PCR product is the expected one. Hence, the product was also gel-purified and sequenced. The sequence obtained is shown in figure 4.8, and was found to be identical to the expected product, except for a single base, which is likely to be due to the taq DNA polymerase being imprecise.

Figure 4.5: PCR using RNA instead of cDNA



lane 1 = 123 bp ladder

lane 2 = ASC 1+2

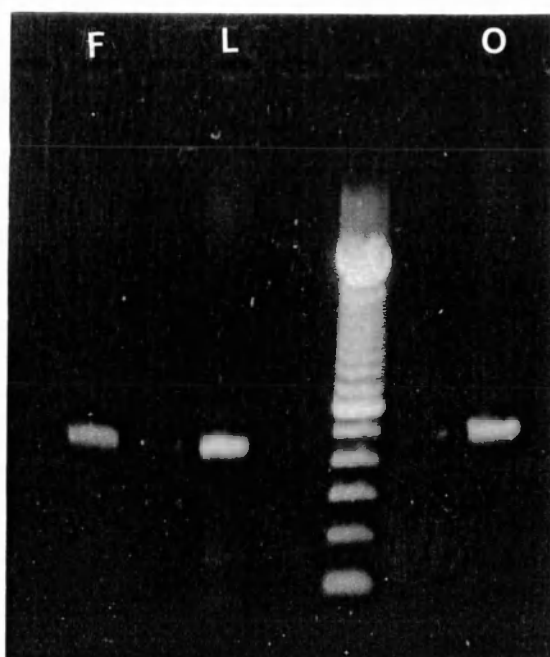
lane 3 = ASC 2+3

lane 4 = β -gluc

lane 5 = GAPdH

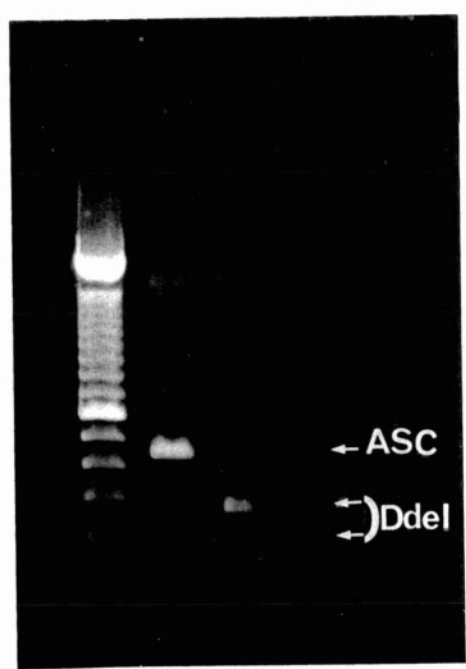
PCR was carried out on untreated RNA instead of cDNA, as described in the text. Any bands obtained must result from the presence of contaminating genomic DNA.

Figure 4.6: PCR Products for Transport System ASC



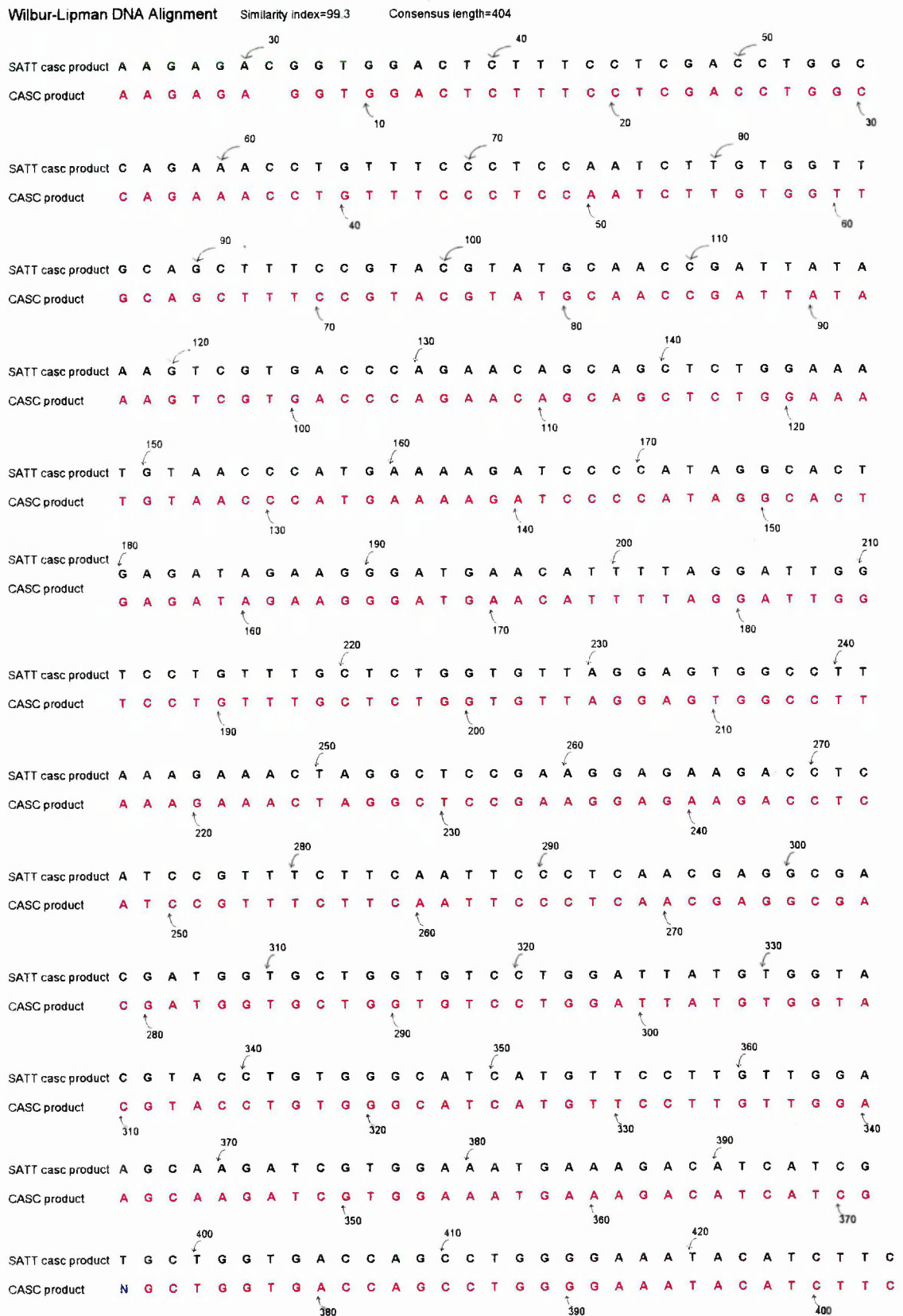
PCR was carried out using specific primers for the ASC gene, as described in the text. Follicles (F), ORS cells (O) and LSDM3 cells (L) were all found to express the ASC gene, giving a PCR product of between 400 and 500 bp in length.

Figure 4.7: Restriction Enzyme digestion of PCR products for ASC



The ORS PCR products for ASC were digested with the restriction endonuclease DdeI as described in the text. This resulted in two fragments of approximately 180 and 280 bp respectively.

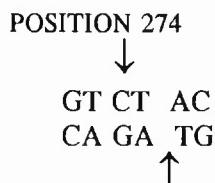
Figure 4.8: Sequence of ASC PCR product



The PCR products for ASC were gel-purified and sequenced as described in the text. The figure shows the sequence of the PCR product alongside the sequence of the expected product from the known sequence of the gene

4.5 PCR of System y^+

Follicles (F), ORS cells (O) and LSDM3 cells (L) were also all found to express the CAT-1 gene for system y^+ . The restriction enzyme *AccI* was found to cut the 400bp product once, to give fragments of about 127 and 274 bp as expected (Figure 4.9).



As with ASC, sequencing of the PCR product confirmed that it was the y^+ gene (figure 4.10).

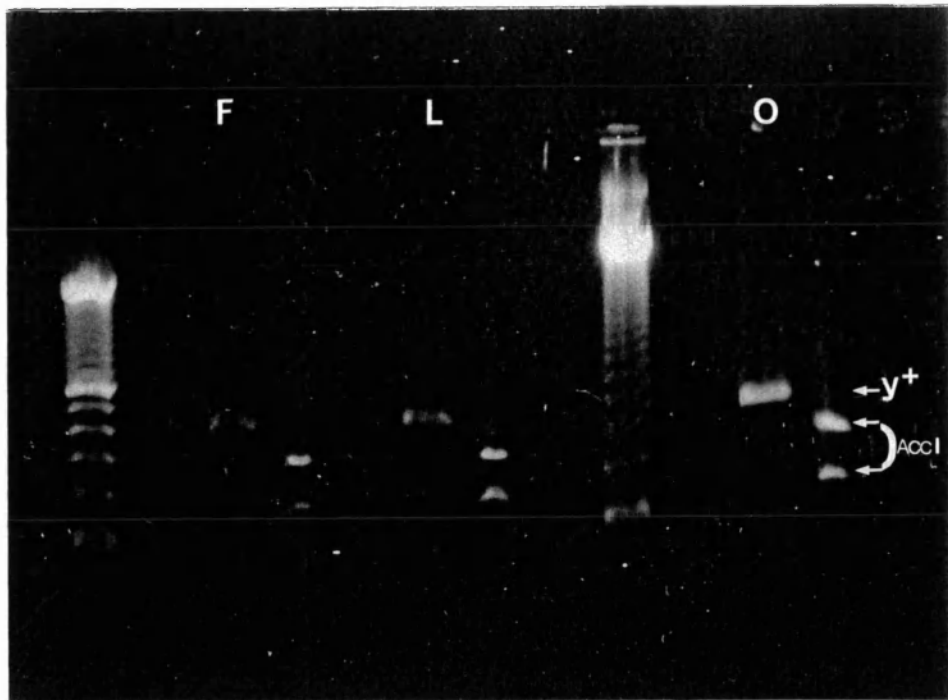
4.6 PCR of rBAT

rBAT (expected product size 586bp) was not detected by PCR (figure 4.11). This may be because the primers do not bind specifically to the rBAT gene, but since no bands were obtained, it is more likely that the gene is not expressed in these cells or tissues. (A non-specific primer pair would give rise to multiple bands.)

4.7 PCR of PepT1 and HPT-1

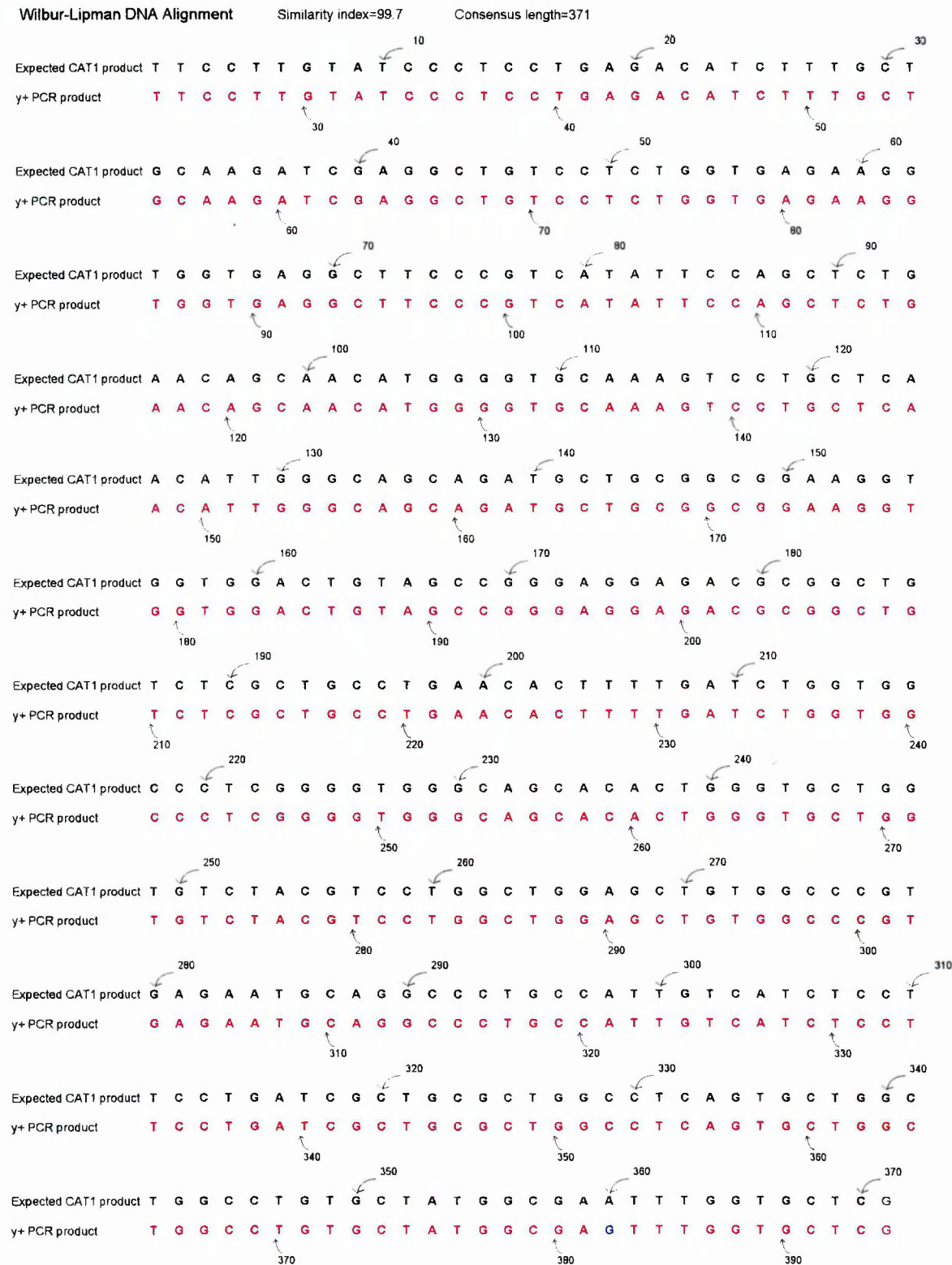
Neither PepT1 (expected product size 416bp) nor HPT-1 (expected product size 414bp) were detected by PCR. A few faint bands were visible (figure 4.12) so the primers were tested on a gut cell-line to see if they would detect the genes in cells that might be expected to express the products. However, the gene products were not detected in this cell-line either (figure 4.13), so the results are inconclusive.

Figure 4.9: PCR products and their restriction enzyme digests, for system y^+



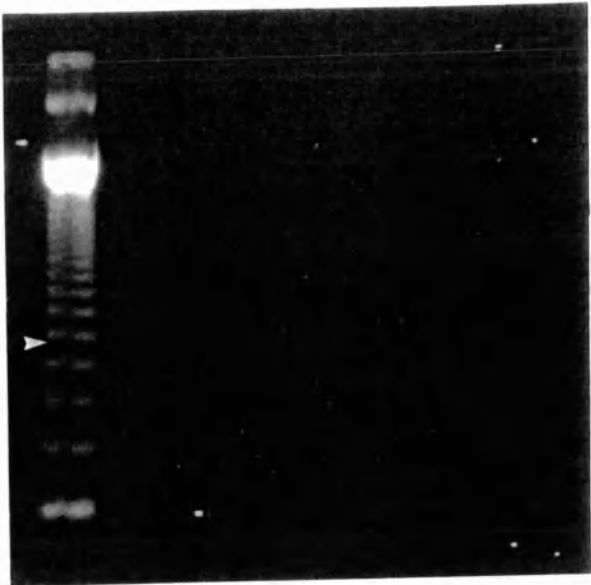
PCR was carried out using specific primers for the y^+ gene, as described in the text. Follicles (F), ORS cells (O) and LSDM3 cells (L) were all found to express the y^+ gene, giving a PCR product of 401 bp in length. The PCR products for y^+ were digested with the restriction endonuclease *AccI* as described in the text. This resulted in two fragments of 127 and 274 bp respectively.

Figure 4.10: Sequence of the y+ PCR product



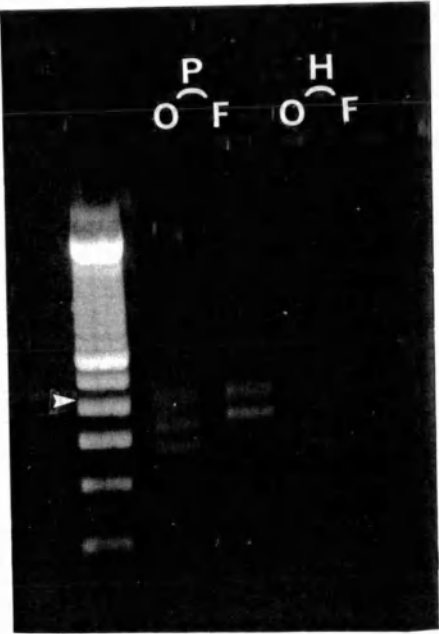
The PCR products for y+ were gel-purified and sequenced as described in the text. The figure shows the sequence of the PCR product alongside the sequence of the expected product from the known sequence of the gene.

Figure 4.11: PCR to detect rBAT



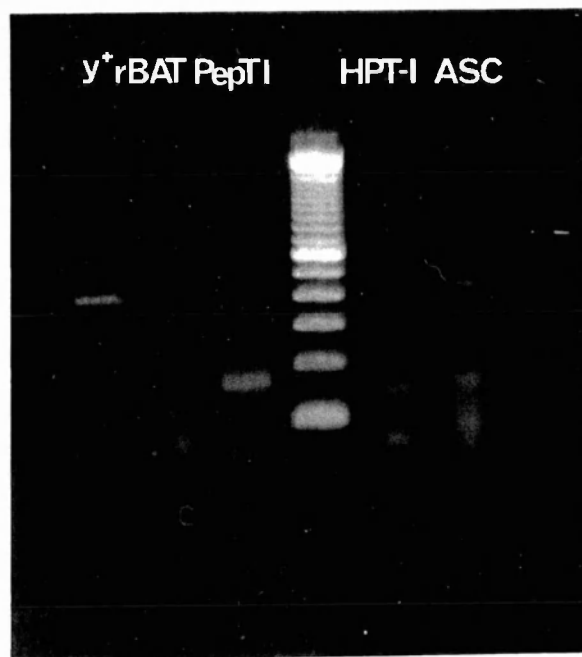
PCR was carried out using specific primers for the rBAT gene, as described in the text. No PCR products were obtained (Arrowhead shows expected product size, 586 bp). Lane 1 = 123 bp ladder, lane 3 = ORS, lane 5 = LSDM3, lane 7 = follicles. Lanes 2, 4 & 6 are empty.

Figure 4.12: PCR to detect peptide transporters PepT1 and HPT-1



PCR was carried out using specific primers for the PepT1 (P) and HPT-1 (H) genes, as described in the text. No major amplified products were obtained, although a few faint bands were visible. (Arrowhead shows expected product size, 414-416 bp.)

Figure 4.13: PCR to detect PepT1 and HPT-1 in intestinal cell line INT407



PCR was carried out on the gut cell line, INT407, using specific primers for the PepT1 (P) and HPT-1 (H) genes, as described in the text. A faint band was obtained for PepT1, but it was 100-200 bp, so it was not the expected product (416 bp). PCR products for y^+ and ASC, but not rBAT, were obtained from INT407 cells.

4.8 Discussion

This study has identified for the first time that follicles, outer root sheath cells and LSDM3 cells each express the amino acid transporters ASC (ASCT1/SATT) and y^+ (mCAT1), but suggests that the peptide transporters, PepT1 and HPT-1, and the putative transport subunit/activator encoded by rBAT are not expressed.

This similarity between the cells/follicles studied justifies using ORS cells, or the LSDM3 cell-line if insufficient ORS cells can be grown, to carry out functional studies of amino acid transport. However, there may be differences in transporters other than those looked for here, so ideally studies should be done on ORS cells rather than LSDM3 cells, and the ultimate goal will be to develop methods to investigate follicles, to examine zonal uptake of amino acids.

The presence of the gradient-driven transport systems ASC for cysteine and y^+ for arginine is consistent with the view that these amino acids are important to hair follicles. These transporters will enable follicles to actively scavenge these key amino acids from the blood supply, and accumulate them against their concentration gradients. There is likely to be additional transport by other transporters, *e.g.* System L as well as system ASC is involved in cysteine transport in erythrocytes (*Tunncliffe, 1994*).

System ASC is widespread, but it has not previously been shown to be expressed in hair or skin. System ASC is believed to be usually non-inducible (*McGivan & Pastor-Anglada, 1994*) although in endotoxemia-stimulated amino acid consumption by the liver, a 3-fold increase in system ASC transport activity occurs, secondary to an increase in carrier maximum velocity, mediated largely by the cytokine tumour necrosis factor (TNF). Increases in the activity of system A (6-fold) and system N (3.5-fold) also occur in this situation, however, so ASC is not of prime importance in this

regulation (*Inoue et al., 1994*). Thus, system ASC in hair follicles and ORS cells may not necessarily be regulated at the transcriptional level. Other processes, such as *trans*-stimulation and competition, may well be important, however. Unlike leucine which appears from autoradiographs to be taken up from the follicle bulb (see Chapter III), it has been shown recently (*Philpott, personal communication*) that cysteine is taken up and incorporated into hair proteins specifically at the zone of keratinization. Therefore, it is possible that expression of system ASC may vary along the length of the hair follicle, perhaps being expressed specifically in the keratinising zone. *In situ* work, or PCR on follicle pieces from different regions of the follicle, would help to characterise this "zonal uptake" with respect to location and function of cysteine transporter(s). Zonal uptake has also been reported in sheep (*Philpott, personal communication*).

In tissues that have been studied previously, such as liver and kidney, system y^+ transports basic amino acids, such as arginine, lysine, histidine and ornithine. The transport is not dependent on the presence of Na^+ , but it is electrogenic, *i.e.* it is driven by the membrane potential. The negative resting potential allows cells to accumulate cationic amino acids against their concentration gradient. mCAT1 is widely-distributed, although it is not ubiquitous, *e.g.* it is not found in the liver (*Kilberg et al., 1993*). The liver expresses mCAT2a instead, which has a lower substrate affinity (*MacLeod et al., 1991*). In liver, glucagon treatment has been shown to stimulate system y^+ activity (*Handlogten & Kilberg, 1984*). It would be interesting to see if mCAT2 is also expressed in ORS cells and hair follicles. In any case, the presence of system y^+ in follicles means that there is an active (membrane-potential driven), high affinity (mCAT1) uptake system for arginine, which is likely to be competitively inhibited by lysine or histidine. Any molecule that alters the cell membrane potential is also likely

to influence arginine uptake by system y^+ . Uptake by system y^+ is subject to *trans*-stimulation when intracellular substrate concentrations are sufficiently high (*White & Christensen, 1982b*). This is partly because the translocation of the substrate-loaded form of the carrier across the plasma membrane is the rate-limiting step of transport. Arginine transport has been shown to be the rate-limiting step for arginine metabolism by arginase in liver (*White & Christensen, 1982a*), and in erythrocytes (*Kuchel et al., 1984*). Thus, system y^+ may represent an important regulatory step in arginine metabolism by hair follicles.

If system y^+L is also present in hair follicles, this will co-transport a neutral amino acid plus a Na^+ ion, as well as basic amino acids. Therefore, neutral amino acids in the presence of Na^+ can competitively inhibit system y^+L and exchange with basic amino acids (*Eleno et al., 1994*). Interestingly, binding but not translocation seems to be sensitive to the structure of the amino acid being transported, e.g. glutamate binds very weakly, but it can cross the membrane on y^+L almost as quickly as lysine (*Angelo & Devés, 1994*). This suggests that it is not driven by membrane potential in the same way as system y^+ , and may support facilitated diffusion rather than active transport. Consistent with this hypothesis is the finding of *Eleno et al. (1994)* that y^+L is not greatly affected by membrane potential. In any case, the presence of y^+L in follicles will have to be determined by functional measurements of amino acid uptake, as the gene sequence is not yet available.

The failure to detect the peptide transporters PepT1 and HPT-1 suggests that they are not expressed in hair follicles. However, the possibility that the PCR primers used may not have been of sufficiently high affinity or specificity for the genes cannot be ruled out. The positive control cell-line used, INT407, of jejunal origin, is very

likely to have adapted significantly to culture conditions, and it is not known that the cells normally express these peptide transporters, so the absence of PCR products with INT407 does not prove that the primers are inadequate. Actual gut tissue would be a much better positive control, but was not available. PepT1 is predicted to be a 12-transmembrane domain protein, and its mRNA has been found in small intestine, kidney and liver, and small amounts in brain, but not in heart, spleen or large intestine (*Fei et al., 1994*). Functional studies have since established that the renal peptide transport system is similar but not identical to its intestinal counterpart, and the renal gene has now been cloned and designated PepT2 (*Liu et al., 1995*). A next step would therefore be to look for PepT2 in hair follicles. The HPT-1 gene product, in contrast, is predicted to have only a single transmembrane domain, and as it gave only a small rise (2-3 fold) in transport when expressed in Chinese hamster ovary cells (*Dantzig et al., 1994*), it is not clear whether HPT-1 is actually a transporter. *Dantzig et al. (1994)* did not detect HPT-1 expression in skin, consistent with its apparent lack of expression in hair follicles observed here. It may be that hair follicles lack the systems required for uptake of peptides, perhaps because they do not normally encounter significant quantities of peptides in the blood, and it may be more efficient for them to take up amino acids which can be metabolised directly, rather than taking up peptides which then have to be broken down to their constituent amino acids before being utilised. Whereas, in gut, kidney and liver there is clearly a requirement for uptake of peptides, as the end-products of protein digestion or (re)absorption of endogenous serum peptides, and also in brain, where certain peptides, such as somatostatin, act as neurotransmitters. However, it is possible that follicles do express peptide transporters other than PepT1 and HPT-1, so the PepT2 gene should be probed for, and functional transport of

radiolabelled peptides measured, before it can be stated conclusively that peptides are not substrates for follicle transport mechanisms.

Since no PCR products were obtained corresponding to rBAT, it seems that this gene is not expressed in ORS cells or hair follicles. Previously, the rBAT protein has been found in the kidney and small intestine (*Palacín, 1994*), and it appears to be important for cystine transport, as described in section 1.3.4. Another gene, 4F2hc, is homologous (30% amino acid identity, 50% similarity) to rBAT, but it involves a different, system y^+L -like transport in *Xenopus* oocytes (*Fei et al., 1995*). It is possible that there are more members of this family of putative transport subunits or activators. It remains a mystery how they participate in amino acid transport although it seems that rBAT is linked by disulphide bridges to an unidentified putative "light subunit" in rat kidney (*Furriols et al., 1993*), so perhaps this subunit has to be present for rBAT to function. Since the role of rBAT is unclear, its absence in hair follicles and ORS cells has no obvious implications at present. It would be worth looking for 4F2hc expression, as system y^+L has now been identified in an epithelial cell-line (*Eleno et al., 1994*), and it does have a high affinity for arginine, and takes part in exchange reactions, which could be important in hair follicles. However, functional studies with arginine (putative 4F2hc substrate) and cystine (putative rBAT substrate) will help to characterise the transport of these important amino acids in a more direct way.

The study of amino acid transport at the molecular level is a relatively new area of research, and it is most likely that more transport systems will soon be cloned, and new systems identified, which may be of considerable importance to the hair follicle.

CHAPTER V

MEASUREMENT OF AMINO ACID TRANSPORT

5.1 Introduction

The purpose of this study was to investigate the uptake of amino acids by outer root sheath (ORS) cells, in order to see how follicles take up these important nutrients, and to see whether this uptake can be manipulated. Expression of the genes for the amino acid transporter systems ASC and y^+ has already been demonstrated as described in chapter IV. These transporters transport cysteine and arginine respectively. However, many transport systems exist, with overlapping specificities, such that a particular amino acid will be a substrate for more than one system. For example, arginine may be transported by system y^+L as well as by system y^+ , and this will have implications for its transport rate and regulation. Therefore, in order to understand how follicles transport a given amino acid, and how this transport is regulated and interacts with other solutes, it is necessary to measure the uptake directly.

In this study, it was decided to look at cells rather than whole follicles, since whole follicles contain considerable interstitial space which may trap the labelled amino acid, giving falsely-high uptake measurements, and the multiple cell types present may give complex results that are difficult to interpret. ORS cells were chosen, as they are the first cells encountered by amino acids coming to the hair follicle from the blood (or medium *in vitro*). LSDM3, an immortalised human keratinocyte cell-line derived from a tumour, was used for method development, to avoid the time and expense required to obtain large numbers of ORS cells. The suitability of LSDM3 cells as a model was

confirmed previously (chapter IV), insofar as they express the same transporters as ORS cells at the genetic level, at least for those transporters studied.

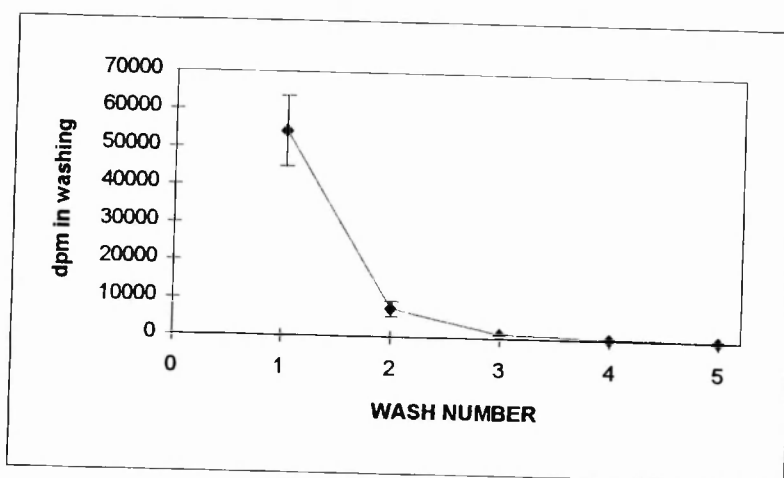
The present findings have determined the relative transport rates of arginine, glutamate, serine/cysteine and MeAIB (2-methylaminoisobutyric acid), and indicated whether or not they are driven by Na^+ gradients or membrane potential. In addition, the results demonstrate that certain amino acid combinations would be undesirable if maximal uptake of one amino acid is required, since those amino acids compete for the same transport system. Further work is required to find other modulators of transport, such as hormones or drugs.

5.2 Method Development

Initial experiments were carried out to identify the number of washes required to remove non-specifically bound radioactivity from test cells. Figure 5.1 shows that five "stop solution" washes were found to be sufficient to remove excess extracellular labelled substrate following an assay for amino acid transport.

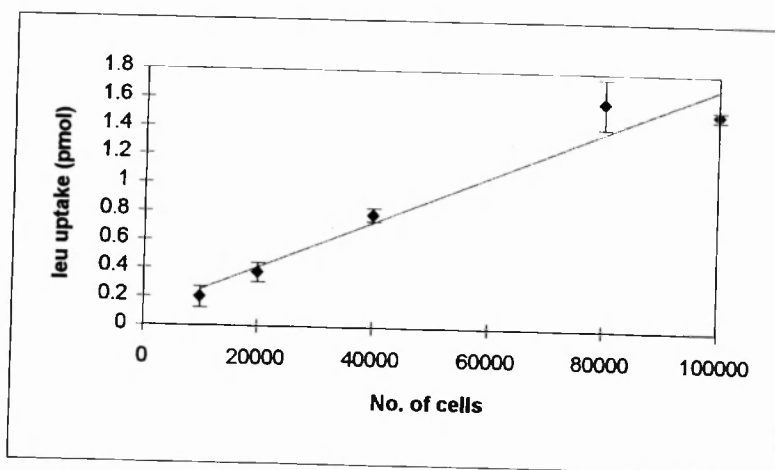
As described in chapter II, section 2.10, the variation in cell number between different wells was measured by labelling the cells with ^3H -leucine for about 1 hour prior to the transport experiments. To verify this method of estimating cell number, the change in ^3H -leucine labelling with increasing cell number was measured. Figure 5.2 shows that the ^3H -leucine labelling increases linearly with cell number up to at least 10^5 cells.

Figure 5.1: Effect of Washing 3H-sucrose-labelled monolayers



Cells were labelled for 3 minutes with 0.6 μ Ci/well 3H-sucrose, then washed with successive aliquots of 0.5ml "stop solution", as described in the text. The radioactivity in the washings was counted. Results show the mean \pm SD of n=3 wells per wash number.

Figure 5.2: 3H-leucine Labelling vs Cell Number



LSDM3 cells were seeded onto 12-well plates at various densities, then labelled for 1.5 h with 148 MBq/mmol 3H-leucine, then washed x 3 with 0.5ml "stop solution", as described in the text. Results show the mean \pm SD radioactivity in the cells for n=3 wells per cell number.

5.3 Measurement of Intracellular Volume

5.3.1 Intracellular Volume of LSDM3 cells

In an experiment where cells from 9 wells of a 12 well plate were measured (as described in chapter II), the mean intracellular volume was 1.01 μl per well. The number of cells was counted in 8 wells and found to be approximately 1.6×10^5 cells per well. Thus, the volume of one LSDM3 cell is approximately 6.3 pl. A repeat of this experiment gave values of 0.34 μl per well and 2.4 pl for the volume of a single cell. A third repeat gave values of 0.22 μl per well and 1.54 pl for the volume of a single cell. A fourth measurement established the volumes as 0.16 μl per well and 1.44 pl for the volume of a single cell. Finally, a fifth measurement established the volumes as 0.5 μl per well and 1.5 pl for the volume of a single cell. The problem with these measurements is that the intracellular volume is small compared with the extracellular volume, so that the calculation involves subtracting a large number from a slightly larger number, to yield a small difference. The standard deviation of the two volume measurement thus tends to mask the small difference between them, giving a very approximate answer. Therefore, an accurate volume cannot be stated, but it is likely that the total intracellular volume in a sub-confluent well of a 12 well plate is approximately between 0.16 and 1.01 μl .

The purpose of this measurement is to determine whether cells are accumulating an amino acid against its concentration gradient, or whether the amino acid is merely diffusing across the cell membrane to reach equilibrium. An approximate value of intracellular volume will enable this assessment to be made, but a more accurate method of measuring cell volume is clearly required.

Sample calculation:

$$\begin{aligned} &^3\text{H-sucrose dpm added in 0.5 ml} = 2.39 \times 10^6 \\ &\text{mean } ^3\text{H-sucrose dpm of sample} = 2.15 \times 10^4 \pm 1.5 \times 10^3 \\ &\therefore \text{sucrose volume of sample (extracellular)} = 2.15 \times 10^4 / 2.39 \times 10^6 \times 0.5\text{ml} = 4.50\mu\text{l} \end{aligned}$$

$$\begin{aligned} &^{14}\text{C-2-deoxyglucose dpm added in 0.5 ml} = 2.36 \times 10^5 \\ &\text{mean } ^{14}\text{C-2-deoxyglucose dpm of sample} = 2.20 \times 10^3 \pm 1.65 \times 10^2 \\ &\therefore \text{2-deoxyglucose volume of sample (total)} = 2.20 \times 10^3 / 2.36 \times 10^5 \times 0.5\text{ml} \\ &= 4.66 \mu\text{l} \end{aligned}$$

$$\therefore \text{Intracellular volume per well} = 4.66 \mu\text{l} - 4.50 \mu\text{l} = 0.16 \mu\text{l}$$

$$\text{Number of cells per well} = 1.11 \times 10^5$$

$$\therefore \text{volume of 1 cell} = 0.16 / 1.11 \times 10^5 \mu\text{l} = 1.44 \text{ pl.}$$

5.3.2 Intracellular volume of ORS cells

The intracellular volume of ORS cells was measured in the same way, and found to be approximately 0.13 μl per well, and 3.3 pl for the volume of a single cell.

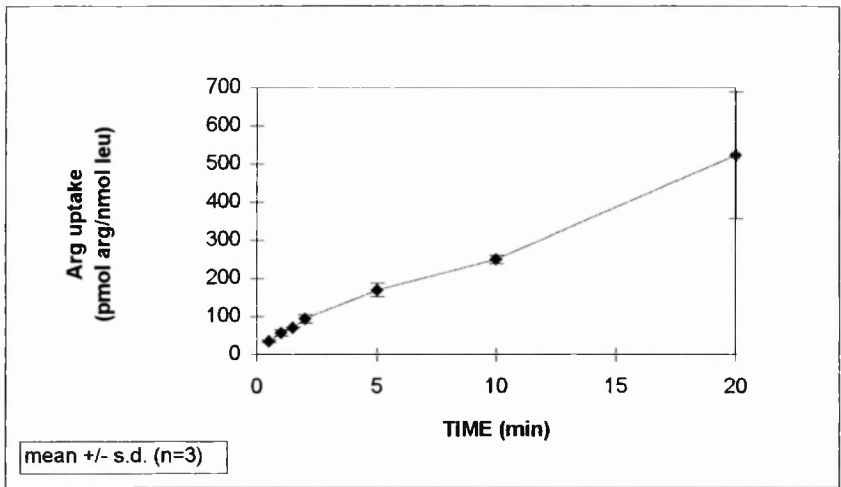
5.4 Transport of Arginine

5.4.1 Uptake of Arginine by LSDM3 cells

5.4.1.1 Time Course

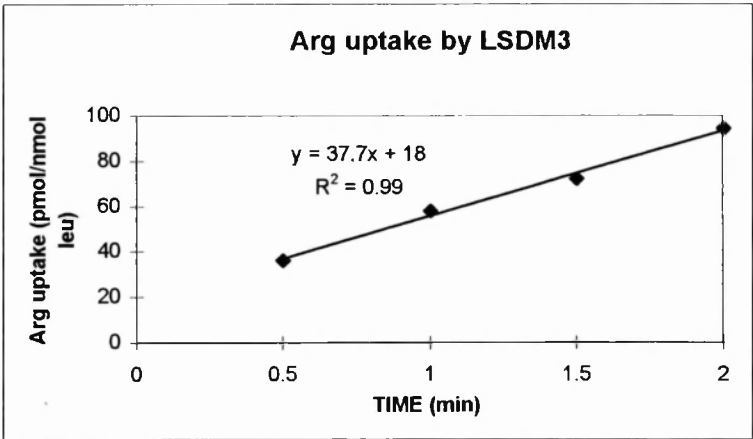
Uptake of 100 μM arginine was measured in 3 separate experiments, using 3 wells per time-point per experiment. Figure 5.3 shows the mean of the 3 experiments. Initial uptake is linear, at a rate of approx. 37.7 pmol / nmol leu / min (figure 5.4), then begins to plateau after about 2 min. The rate of uptake continues to increase by a small extent after 10 minutes, but this may represent incorporation of arginine into protein, or another metabolic process, continuing to drive arginine uptake. Uptake is expected to reach a plateau as the rate of influx of arginine is balanced by efflux, so that eventually there is no net uptake. By extrapolating from Figure 5.4 it may be seen that the intercept with the Y axis is at 18. This suggests that at time zero, some arginine is

Figure 5.3: Time-course of arginine uptake by LSDM3 cells



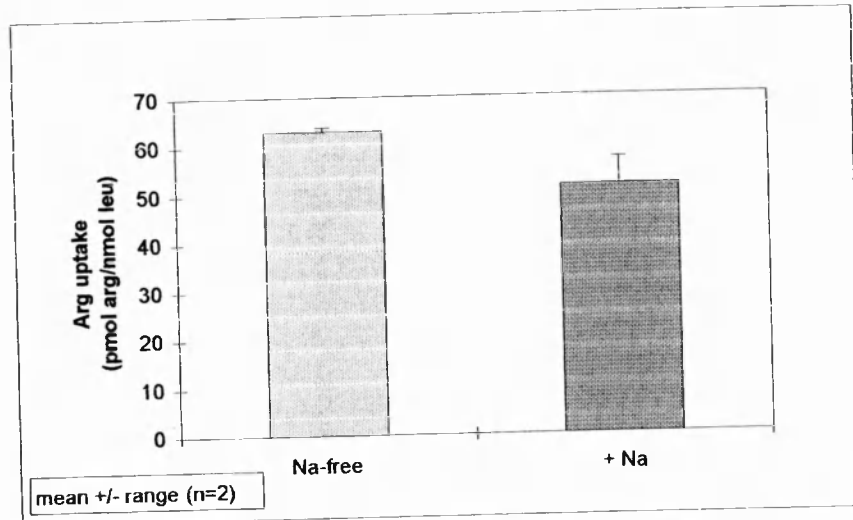
The uptake of ^{14}C -arginine by LSDM3 cells was measured over a time-course of 0 to 20 minutes, as described in the text. The graph shows the mean \pm sem of 3 experiments.

Figure 5.4: Linear uptake of arginine by LSDM3 cells



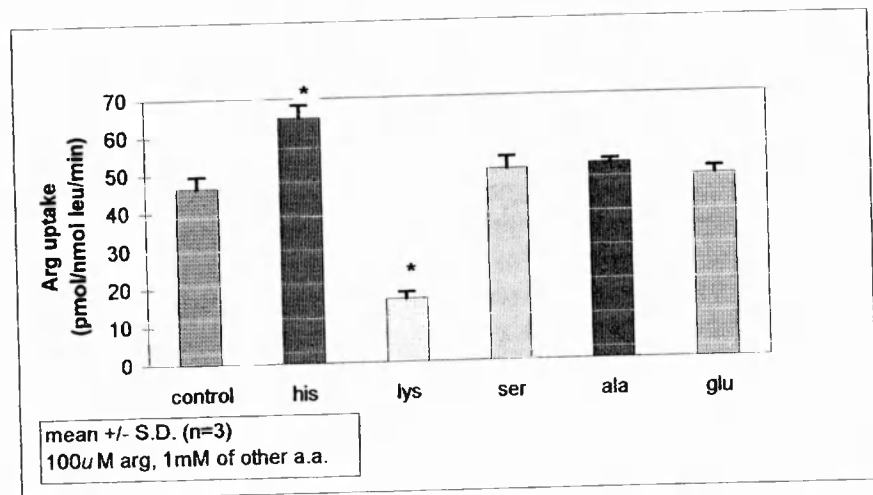
The initial rate of arginine uptake by LSDM3 cells, as shown in figure 5.3 above, was found to be linear ($r=0.99$) with a rate of 37.7 pmol/nmol leu/min.

Figure 5.5: Sodium dependence of arginine transport by LSDM3 cells



The rate of arginine uptake by LSDM3 cells in 1 minute was measured in the presence and absence of sodium, as described in the text. Results show the mean +/- range for 2 experiments with triplicate wells per experiment.

Figure 5.6: Effect of other amino acids on arginine uptake by LSDM3 cells



The rate of arginine (100μM) uptake by LSDM3 cells in 1 minute was measured in the presence of an excess (1mM) of other amino acids, as described in the text. Results show the mean +/- SD for n=3 experiments. *shows significant difference from control ($p < 0.05$) by Student's t test. If the background 18 pmol arg/nmol leu is subtracted from all measurements it can be seen that lysine fully inhibits arg uptake.

already taken up by the cells in the plate. Since this is theoretically impossible, an alternative explanation is that some dead space is present, which is trapping the added arginine such that it is not removed by washing. This could be intercellular space, for example the arginine might adhere to the plastic plate. This value of 18 pmol arg/nmol leu, can be regarded as a background value which should be subtracted from other measurements of arginine uptake.

Since uptake is linear up to 2 minutes, all subsequent experiments were performed using 1 minute incubation periods.

5.4.1.2 Sodium Dependence of Arginine Transport by LSDM3 Cells

Having demonstrated arginine uptake by LSDM3 cells, further experiments were carried out to investigate whether or not this uptake was sodium-dependent. It was found that arginine transport was largely ($81.7 \pm 8.9 \%$) independent of Na^+ ions (figure 5.5).

5.4.1.3 Competitive Inhibition by Other Amino Acids

The effect of an excess of other amino acids (1 mM) on the uptake of 100 μM arginine is shown in figure 5.6. Lysine, but not histidine, serine, alanine or glutamate, was found to inhibit arginine uptake. The apparent increase in arginine uptake seen with excess histidine is actually because there is an efflux of leucine, which means that the ratio of arg/leu is increased. The actual uptake of arginine per well of cells is unaltered.

This ability of histidine to cause leucine efflux was confirmed by a further experiment, summarised in table 5.1, in which D-histidine had no effect on leucine labelling, but L-histidine, which is the biologically active isomer, caused a reduction in

leucine labelling.

Table 5.1: Effect of D-histidine and L-histidine

Sample	Arginine uptake (pmol/well)	Leucine labelling (nmol/well)
Control	293 ± 14.0	7.6 ± 0.1
excess D-histidine	265 ± 11.1	7.4 ± 0.2
excess L-histidine	261 ± 0.9	4.7 ± 0.2 *

(*denotes significant difference from control, according to Student's t-test, $p < 0.05$).

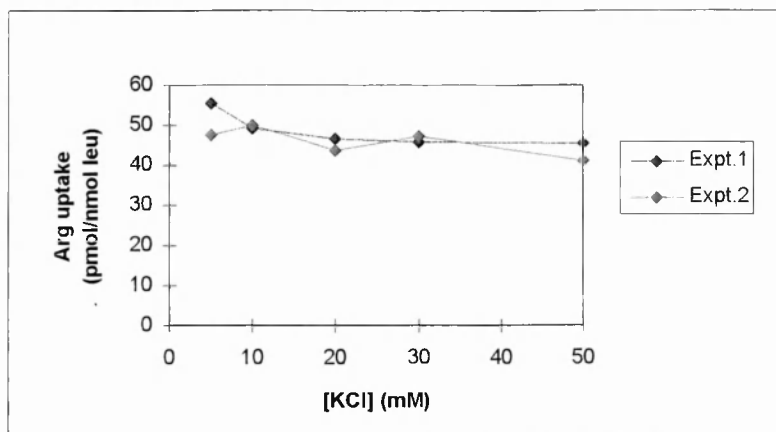
5.4.1.4 Effect of Membrane Potential on Arginine Uptake by LSDM3 Cells

The effect of membrane potential on arginine uptake was investigated, by measuring arginine uptake over a range of external K^+ concentrations. As the external K^+ concentration is increased, the membrane potential should become less negative, and this depolarisation would be expected to decrease the driving force for arginine uptake, since arginine is a cationic amino acid, and system y^+ is known to be regulated by membrane potential in other tissues. Figure 5.7 shows the mean result from 2 experiments, demonstrating a small but insignificant decrease in uptake.

5.4.2 Uptake of Arginine by ORS Cells

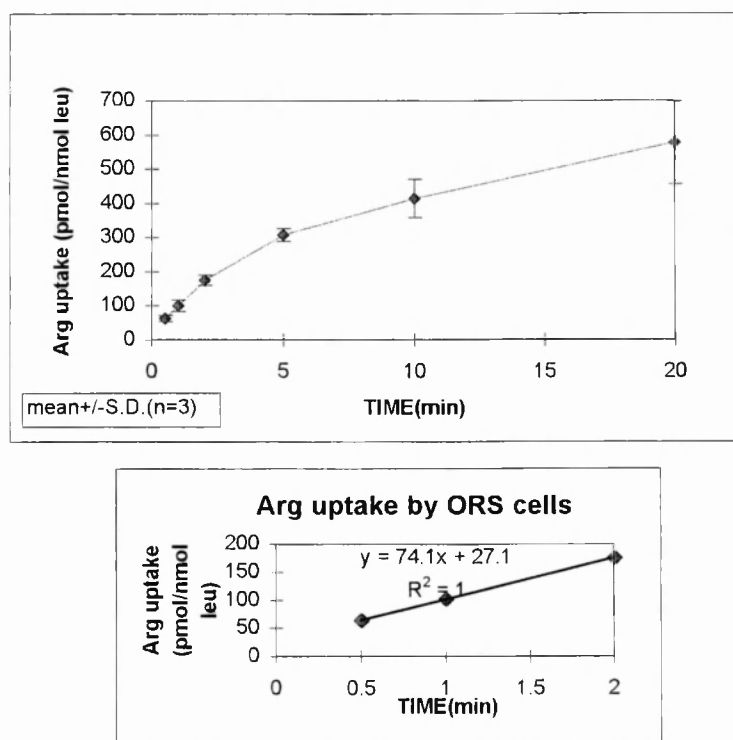
Having established the methods using the LSDM3 cell-line, the experiments were repeated with ORS cells, again repeating each experiment three times on different ORS cell cultures, with triplicate wells per time-point in each experiment.

Figure 5.7: Effect of [KCl] on arginine uptake by LSDM3 cells



The uptake of arginine by LSDM3 cells over a range of potassium ion concentrations was measured as described in the text. The results of two experiments are shown, demonstrating a slight decrease in arginine uptake with increasing [KCl].

Figure 5.8: Time-course of arginine uptake by ORS cells



The uptake of ^{14}C -arginine by ORS cells was measured over a time-course of 0 to 20 minutes, as described in the text. The top graph shows the mean \pm SD of $n=3$ experiments. The lower graph shows the initial linear uptake, at 74 pmol/nmol leu/min.

5.4.2.1 Time Course

As shown in figure 5.8, ORS cells transport arginine with a similar time-course to LSDM3 cells, showing a linear uptake for a couple of minutes after which uptake plateaus. Initial uptake occurs at a rate of approx. 74 pmol / nmol leu / min, compared with a rate of approximately 45 pmol / nmol leu / min for LSDM3 cells. Under the conditions tested, at 100 μ M, arginine reaches a maximum uptake of about 500 pmol per nmol leu, or 1200 pmol per well of cells, in 20 minutes. As was seen with LSDM3 cells, extrapolation from figure 5.8 shows a background value of approximately 27 pmol arg/nmol leu trapped at zero time. This value should, therefore, be subtracted from future measurements of arginine uptake.

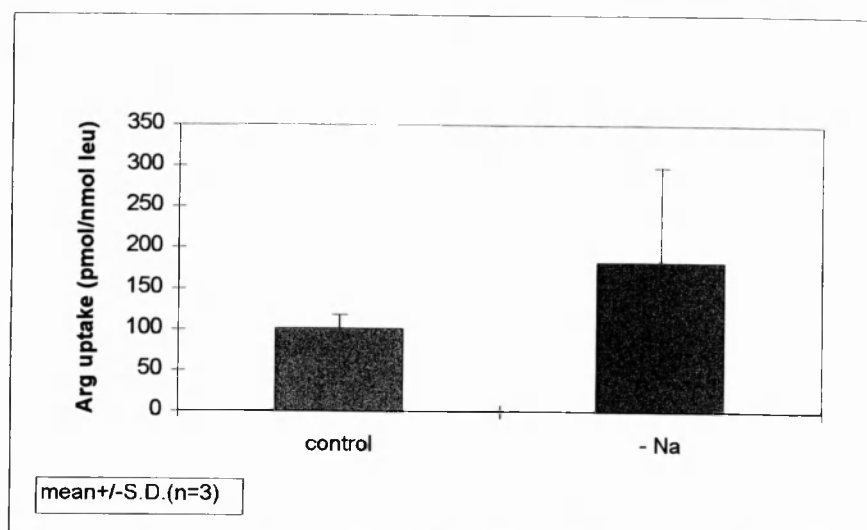
5.4.2.2 Sodium Dependence of Arginine Transport by ORS Cells

As with the LSDM3 cells, a 1 minute time-point was chosen for subsequent experiments, so that regulation of the initial, linear transport rate could be investigated. Figure 5.9 shows that transport of arginine by ORS cells was found to be independent of the presence of Na⁺ ions.

5.4.2.3 Competitive Inhibition by Other Amino Acids

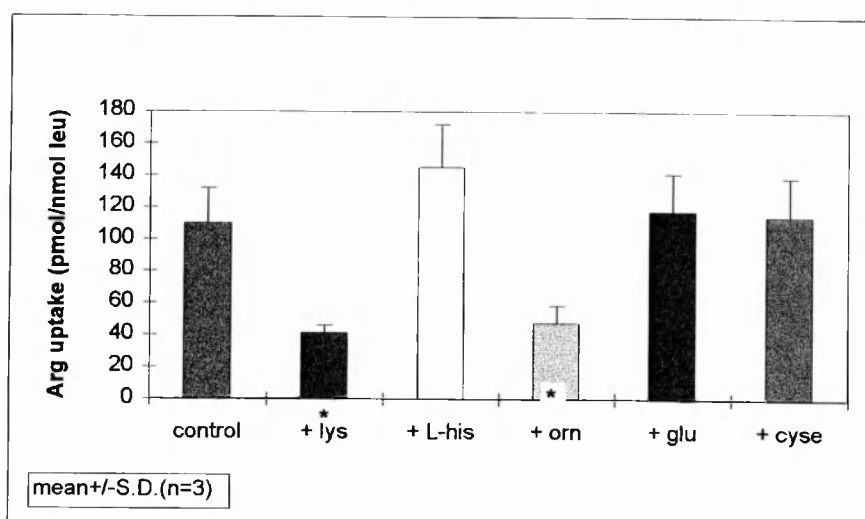
As with LSDM3 cells, the effect of excess other amino acids (1 mM) on the uptake of 100 μ M arginine was measured. Lysine and ornithine, but not histidine, cysteine or glutamate, were found to significantly inhibit arginine uptake ($p < 0.05$), according to Student's t test (Figure 5.10). If the "background" value of 27 pmol arginine trapped in the "dead space" were subtracted from each of these transport measurements, then the inhibition of arginine uptake by lysine and ornithine would be seen more

Figure 5.9: Effect of Na on arginine uptake by ORS cells



Arginine uptake by ORS cells was measured over a 1 minute time period in the presence and absence of sodium ions, as described in the text. Results show the mean \pm SD for n=3 experiments. There is no significant difference between the two rates, according to Student's t test ($p>0.05$).

Figure 5.10: Effect of other amino acids on arginine uptake by ORS cells



The rate of arginine (100 μ M) uptake by ORS cells in 1 minute was measured in the presence of an excess (1mM) of other amino acids, as described in the text. Results show the mean \pm SD for n=3 experiments. *shows significant difference from control ($p<0.05$) by Student's t test. The apparent residual arginine uptake in the presence of excess lys and orn is due to the arginine trapped in "dead space", rather than true uptake.

clearly.

5.5 Transport of Cysteine

5.5.1 Time Course of Uptake of Cysteine by LSDM3 Cells

The transport of cysteine was measured using 20 μM cysteine, in the presence of 2 μM dithiothreitol (DTT) to keep the cysteine in its reduced form. As figure 5.11 illustrates, the transport measurements of cysteine by LSDM3 cells were highly variable, even within the same passage number.

5.5.2 Transport of Serine by LSDM3 Cells.

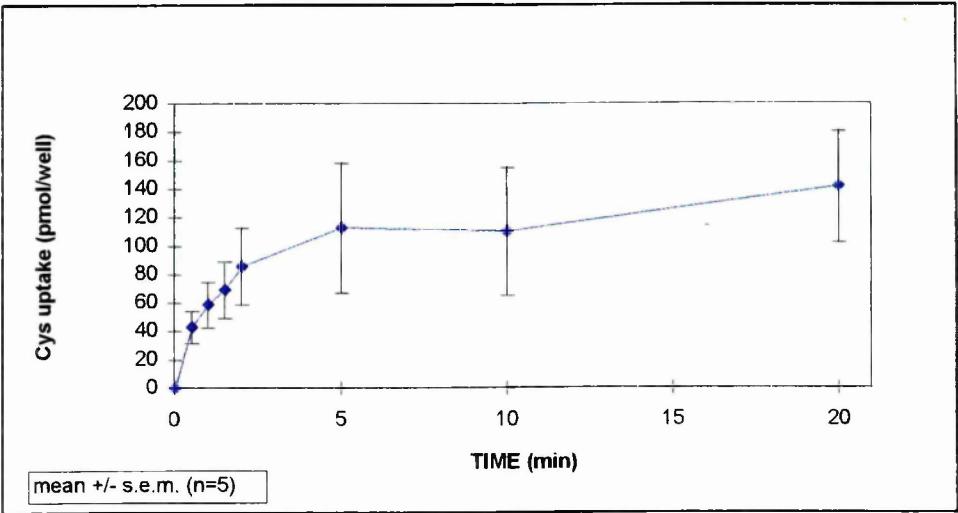
Since cysteine transport varied so much, it was difficult to look at its regulation by Na^+ or other amino acids. Therefore, serine transport was measured (figure 5.12), since serine is also transported by system ASC, and its uptake may mirror that of cysteine. To verify this, cysteine inhibition of serine uptake was demonstrated (figure 5.13). The uptake of serine by LSDM3 cells was found to be almost entirely Na^+ -dependent ($p < 0.005$) (figure 5.14). These results are expressed as per well rather than per nmol leucine, because ^3H -serine was used, because of its availability, so labelling with ^3H -leucine was not carried out. However, this demonstrates the high degree of consistency in the results.

5.6 Transport of Glutamate

5.6.1 Transport of Glutamate by LSDM3 cells

Uptake of 100 μM glutamate over a time-course of 20 minutes showed that LSDM3 cells exhibit only a slow transport of glutamate (5 pmol / nmol leu / min)

Figure 5.11: Cysteine uptake by LSDM3 cells



The uptake of ³⁵S-cysteine by LSDM3 cells was measured over a time-course of 0 to 20 minutes, as described in the text. The graph shows the mean+/-sem of n=5 experiments.

The graph below shows the individual results for the same 5 experiments, revealing the high degree of variation, even within a single passage.

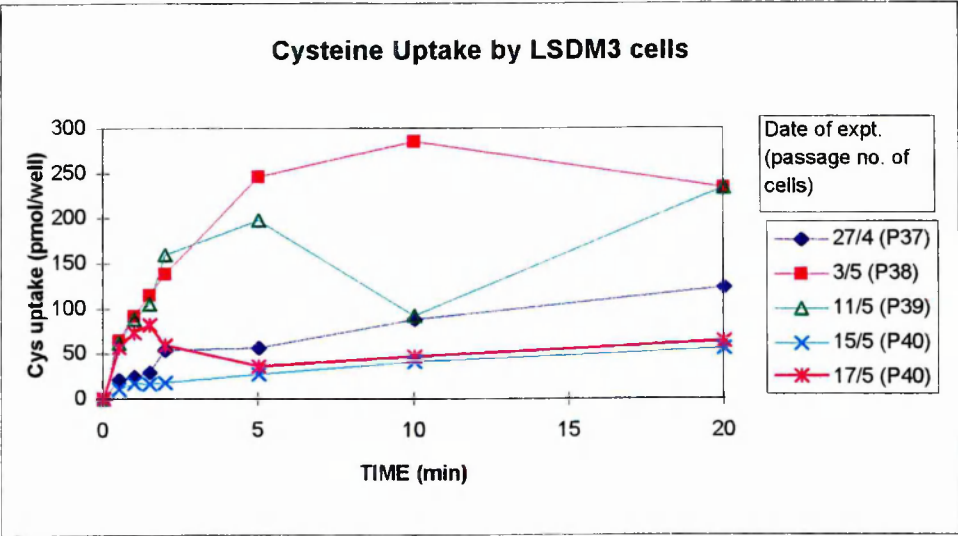
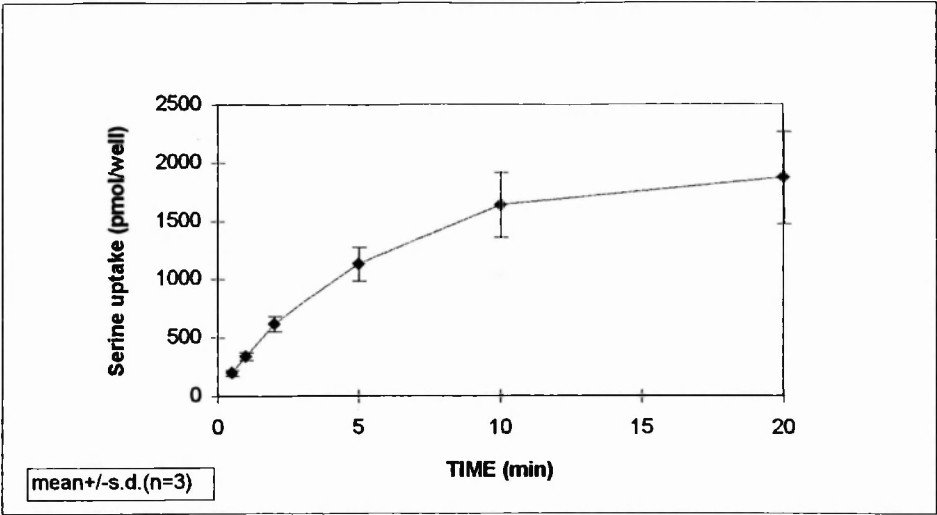


Figure 5.12: Time-course of serine transport by LSDM3 cells



The uptake of 3H-serine by LSDM3 cells was measured over a time-course of 0 to 20 minutes, as described in the text. The graph shows the mean \pm -SD of n=3 experiments.

(figure 5.15), which is largely independent of Na^+ (figure 5.16). Note that, as found with measurement of arginine transport, there appears to be some glutamate "uptake" at time zero, suggesting a background value of approximately 20 pmol glu/nmol leu, which is trapped in the "dead space".

Glutamate uptake did not appear to be significantly affected by excess aspartate, nor by excess cystine either with or without Na^+ ions present (figure 5.17). However, if the background value of 20 pmol glu/ nmol leu is subtracted from these transport measurements, it would appear that aspartate and cysteine do, in fact, inhibit glutamate uptake. Also, it could be concluded that glutamate uptake is actually sodium-dependent. Unfortunately, the large error in the sodium free sample values means that there is no statistically significant difference.

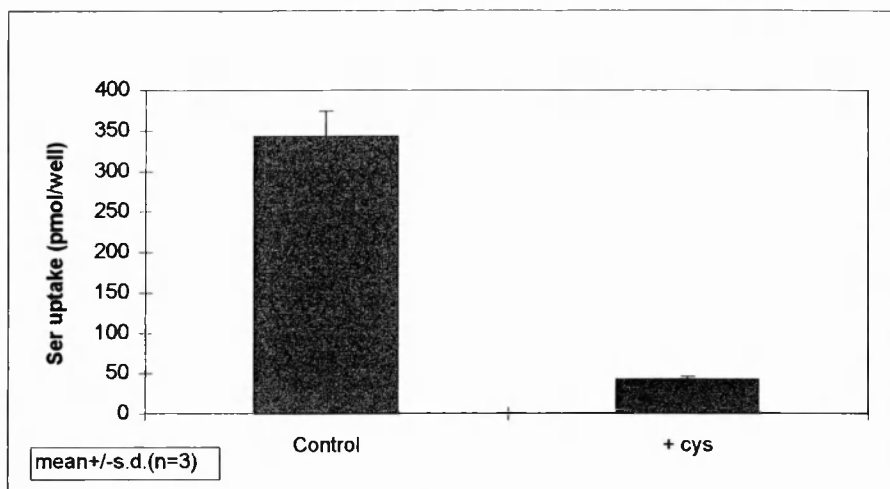
5.6.2 Transport of Glutamate by ORS cells

Uptake of 100 μM glutamate by ORS cells was very similar to that in LSDM3 cells. Transport was quite slow (4 pmol / nmol leu / min) (fig. 5.18), and again there was a background value of about 20 pmol glu/nmol leu at time zero. No significant inhibition was observed in the absence of sodium ions (figure 5.19) or in the presence of excess aspartate or cysteine (figure 5.20), although a proper evaluation of the trapped glutamate in the dead space and subtraction from the uptake data may reveal some differences.

5.7 Transport of MeAIB

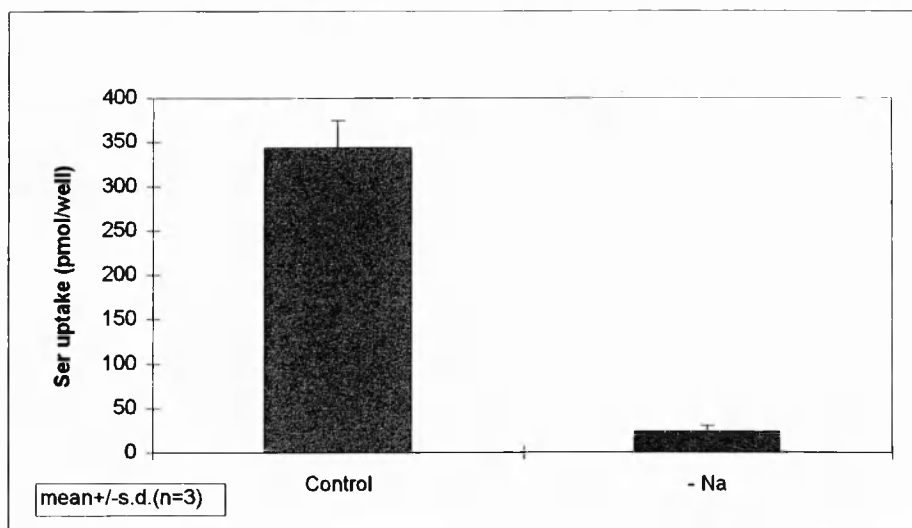
The transport of 2-methylaminoisobutyric acid (MeAIB), a non-metabolisable substrate of system A, was measured, to detect the presence of transport system A.

Figure 5.13: Effect of excess cysteine on serine transport by LSDM3 cells



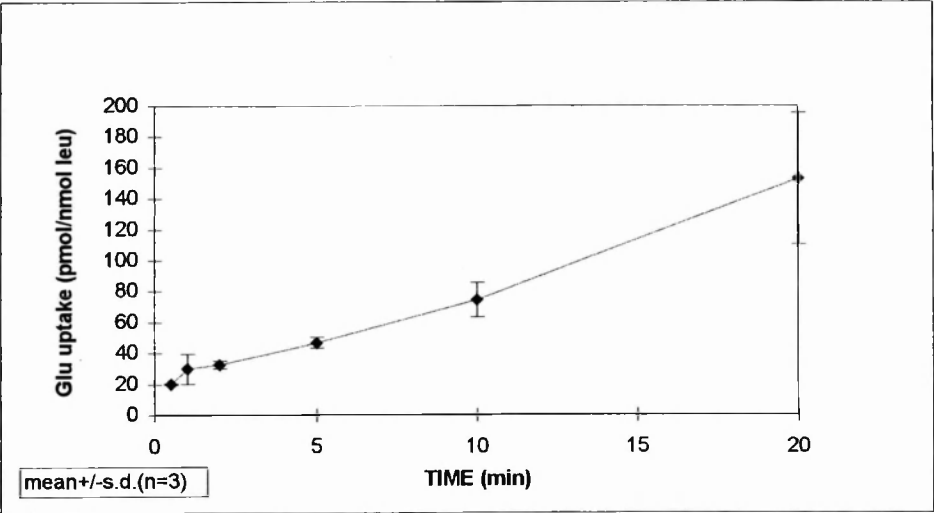
Serine (20 μ M) uptake by LSDM3 cells was measured in the presence and absence of excess (1mM) cysteine, as described in the text. Excess cysteine significantly inhibited serine uptake ($p<0.05$) according to Student's t test. Results show the mean \pm SD for $n=3$ experiments.

Figure 5.14: Sodium dependence of serine transport by LSDM3 cells



Serine (20 μ M) uptake by LSDM3 cells was measured in the presence and absence of sodium ions, as described in the text. Serine uptake was found to be significantly dependent on sodium ($p<0.05$ according to Student's t test). Results show the mean \pm SD for $n=3$ experiments.

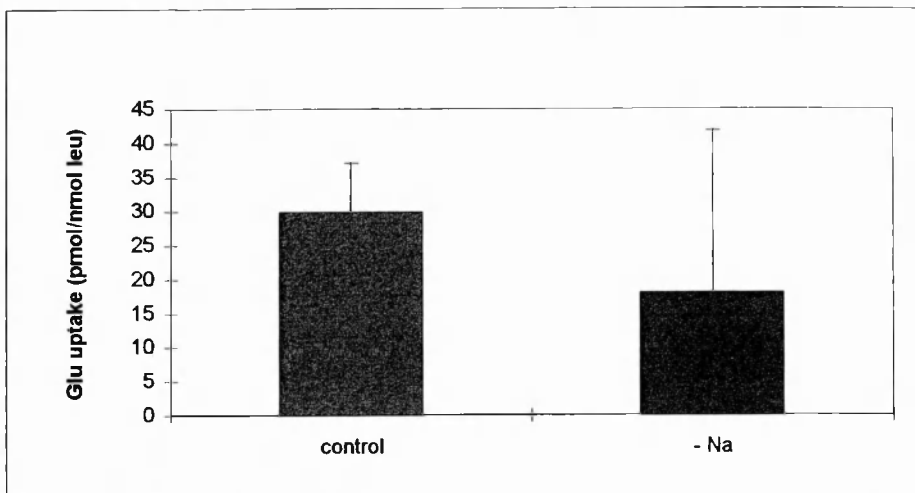
Figure 5.15: Time-course of glutamate uptake by LSDM3 cells



The uptake of ^{14}C -glutamate by LSDM3 cells was measured over a time-course of 0 to 20 minutes, as described in the text. The graph shows the mean \pm SD of $n=3$ experiments.

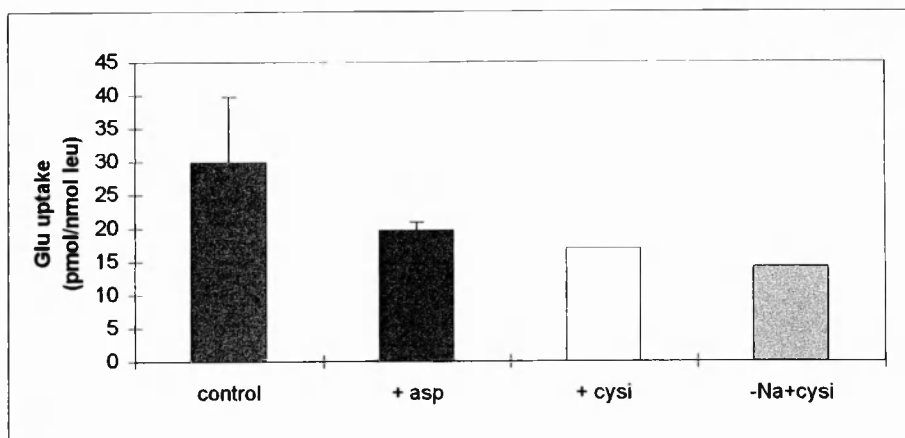
Note the positive intercept with the y axis at time zero, suggesting a background value of approximately 20 pmol glu/nmol leu which is trapped.

Figure 5.16: Na-dependency of Glutamate transport by LSDM3 cells



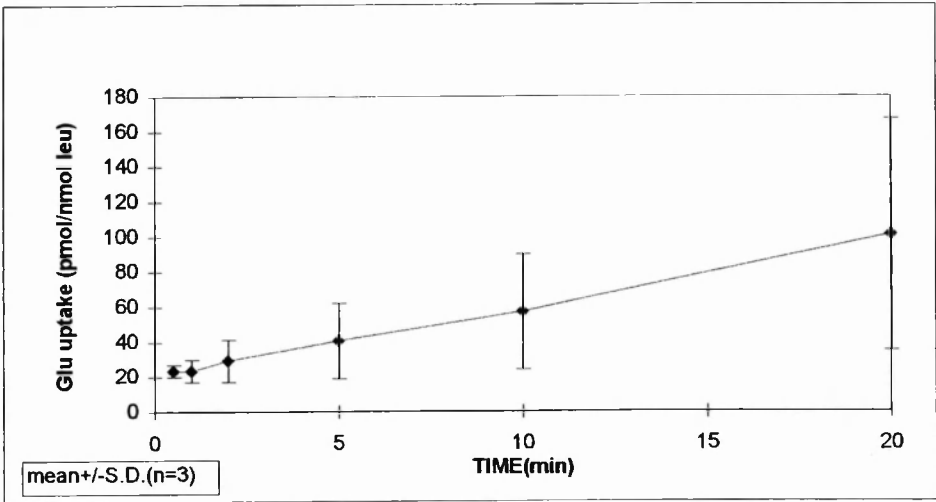
Glutamate (100 μ M) uptake by LSDM3 cells was measured in the presence and absence of sodium ions, as described in the text. Glutamate uptake was found to be unaffected by sodium ($p>0.05$ according to Student's t test). Results show the mean \pm range for 2 experiments.

Figure 5.17: Effect of aspartate and cystine on glutamate uptake by LSDM3 cells



The rate of glutamate (100 μ M) uptake by LSDM3 cells in 1 minute was measured in the presence of an excess (1mM) of other amino acids, with or without Na, as described in the text. Results show the mean \pm SD for $n=3$ experiments.

Figure 5.18: Time-course of glutamate uptake by ORS cells



The uptake of ¹⁴C-glutamate by ORS cells was measured over a time-course of 0 to 20 minutes, as described in the text. The graph above shows the mean +/- SD of n=3 experiments. The lower graph shows the initial linear uptake, at 3.6 pmol/nmol leu/min.

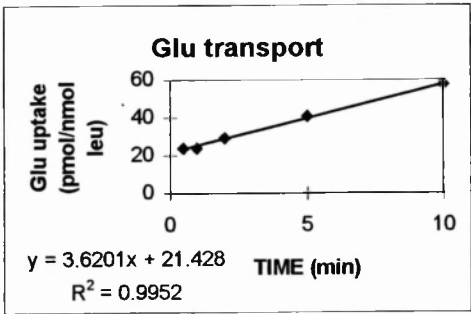
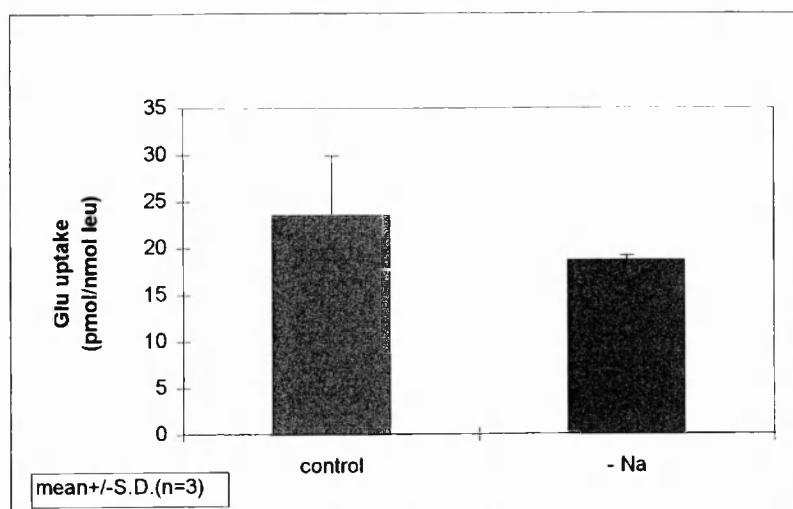
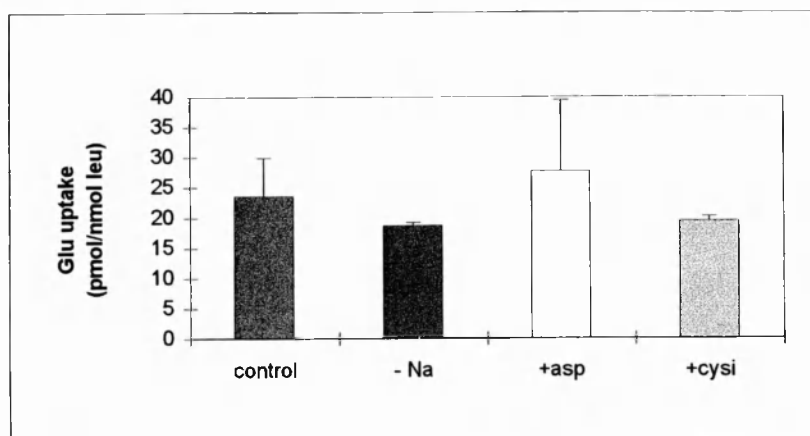


Figure 5.19: Effect of Na on glutamate uptake by ORS cells



Glutamate ($100\mu\text{M}$) uptake by ORS cells was measured in the presence and absence of sodium ions, as described in the text. Glutamate uptake was not significantly inhibited by lack of sodium ($p>0.05$ according to Student's *t* test). Results show the mean \pm SD for $n=3$ experiments.

Figure 5.20: Effect of Na or other amino acids on glutamate uptake by ORS cells



The rate of glutamate ($100\mu\text{M}$) uptake by ORS cells in 1 minute was measured in the presence of an excess (1mM) of other amino acids, with or without Na, as described in the text. Results show the mean \pm SD for $n=3$ experiments.

5.7.1 Time Course of MeAIB Uptake by ORS Cells

As shown in figure 5.21, 0.5 mM MeAIB was transported quite rapidly (approximately 20 pmol / nmol leu / min) by ORS cells, suggesting that system A is present. However, the uptake was only partially (40%) Na⁺-dependent (figure 5.22). Excess cysteine did not appear to affect the transport of MeAIB (figure 5.22). Again, it is possible that some MeAIB has been trapped in the dead space, as extrapolation to the Y axis in figure 5 21 would suggest. This could obscure the effects of sodium or excess cysteine, so it may be that MeAIB is actually fully sodium dependent and inhibitable by excess cysteine, as would be predicted.

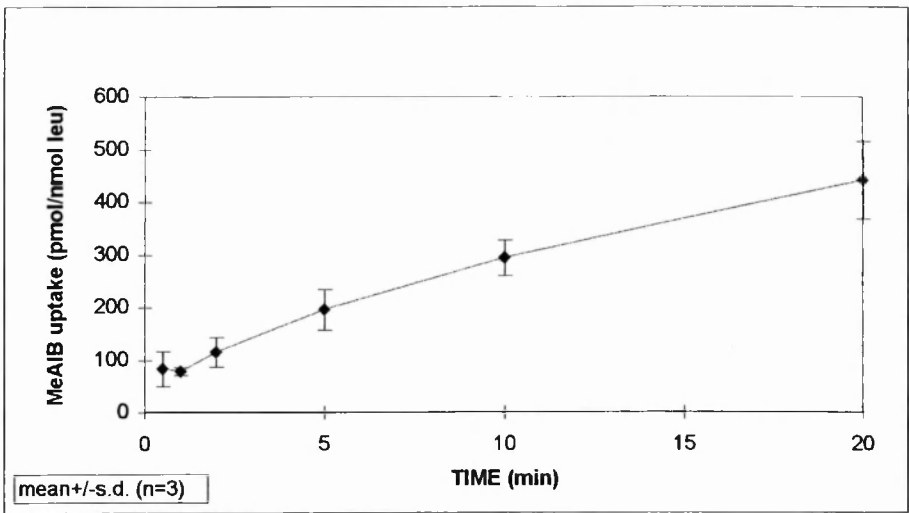
5.8 Effect of Minoxidil on Transport

The hair growth-promoting drug, minoxidil, was tested to see if it had any effect on the transport of arginine or glutamate by ORS cells. Minoxidil was added to the amino acid solution at either 10 or 100 µg/ml final concentration. Cells were pre-incubated with the same concentrations of minoxidil for either zero or 1 hour prior to measuring transport. Preliminary results suggest that minoxidil has no effect on the rate of arginine (figure 5.23) or glutamate (figure 5.24) transport by ORS cells, either with or without pre-incubation.

5.9 Discussion

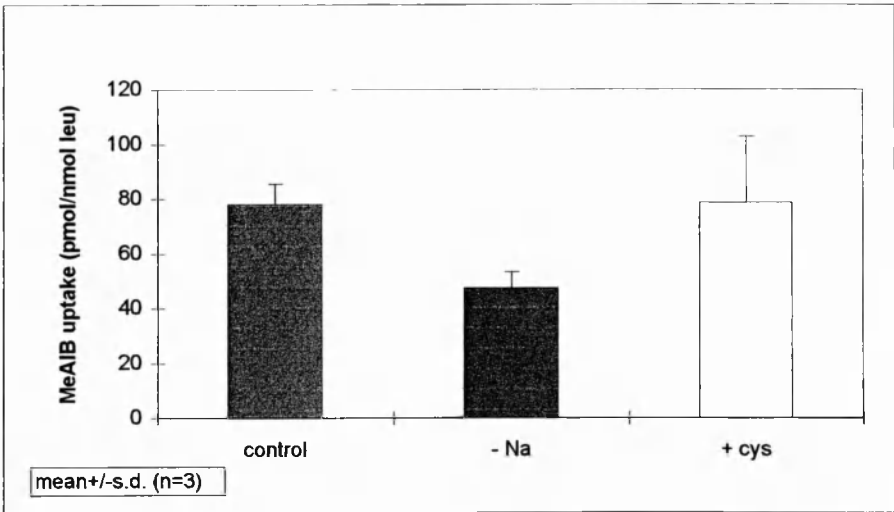
This study is the first to measure the transport of amino acids by human hair follicle ORS cells. It has shown that glutamate uptake by ORS cells is significantly slower than that of arginine, MeAIB or serine, and that of these only serine transport

Figure 5.21: Time-course of MeAIB uptake by ORS cells



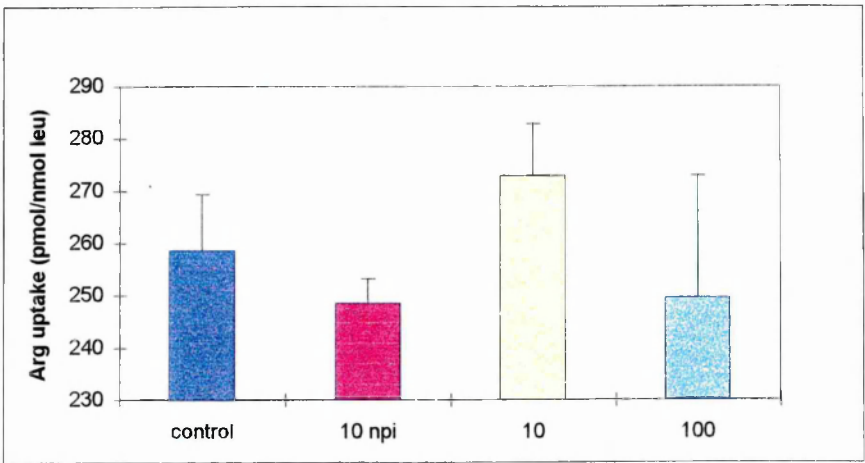
The uptake of ^{14}C -MeAIB by ORS cells was measured over a time-course of 0 to 20 minutes, as described in the text. Results show the mean \pm SD of n=3 experiments.

Figure 5.22: Effect of Na or excess cysteine on MeAIB uptake by ORS cells



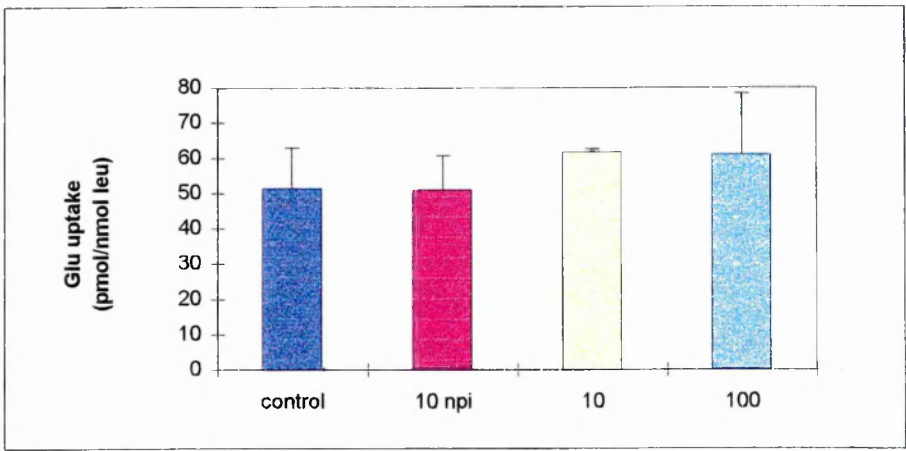
The rate of MeAIB ($500\mu\text{M}$) uptake by ORS cells in 1 minute was measured in the presence of excess (1mM) cysteine, or in the absence of sodium, as described in the text. Results show the mean \pm SD for n=3 experiments.

Figure 5.23: Effect of minoxidil on arginine uptake by ORS cells



Uptake of arginine by ORS cells was measured in the presence of 0 (control), 10 or 100 ug/ml minoxidil. Cells were pre-incubated with the same minoxidil concentrations for 1h prior to measuring transport, except in the samples labelled "npi" (no pre-incubation), to enable both long-term and acute effects to be observed.

Figure 5.24: Effect of minoxidil on glutamate uptake by ORS cells



Uptake of glutamate by ORS cells was measured in the presence of 0 (control), 10 or 100 ug/ml minoxidil. Cells were preincubated with the same concentrations of minoxidil for 1h prior to measuring transport, except in the samples labelled "npi" (no pre-incubation), to enable both long-term and acute effects to be observed.

is completely dependent on the presence of Na^+ ions. It has also identified specific amino acids which compete for transport.

When measuring amino acid transport, it is important to consider the influence of the cell membrane potential on the transmembrane distribution of an amino acid. The cell membrane potential is negative, *e.g.* in cultured keratinocytes the membrane potential is about -24mV (*Mauro et al., 1990*), and this provides a driving force for the entry of positively-charged amino acids to enter the cell, and determines the level of passive distribution across the membrane. If the membrane potential is the only driving force for uptake, a resting membrane potential of -24mV would be expected to result in a 3-fold greater concentration of a positively-charged amino acid inside the cells, according to the Nernst equation. A knowledge of the cells' volume or intracellular water enables the intracellular concentration of an amino acid to be calculated, since concentration equals amount divided by volume. The concentration inside and outside of the cells may then be compared, to see whether the amino acid is actively or passively distributed.

Arginine uptake by ORS cells was found to be rapid (74 pmol / nmol leu / min) and Na^+ -independent. The rate of uptake was not affected by the extracellular presence of excess neutral amino acids such as cysteine, but was significantly decreased by high concentrations of the cationic amino acids lysine and ornithine, although not by histidine. These results are consistent with transport via system y^+ , which transports cationic amino acids such as arginine, ornithine and lysine in a Na^+ -independent manner. Amino acid transport by system y^+ in other cell types, such as endothelial cells, or intestinal epithelial cells, is insensitive to pH, but is driven by the cell membrane potential (*White, 1985*), cell hyperpolarisation increasing the driving force for uptake

of cationic amino acids. However, the arginine uptake measured in LSDM3 cells was only slightly decreased by increasing the external potassium concentration. The low cell membrane potential of keratinocytes suggests that E_K does not contribute more than partially to E_m , and a Na^+ or Cl^- conductance probably plays a significant role in this cell type. Therefore, it is possible that cell membrane depolarisation did not occur over this range of potassium concentration, and this could be tested by measuring the distribution of a lipophilic cation, such as TPP^+ (tetraphenylphosphonium), across the cell membrane at different potassium concentrations. The low membrane potential also implies that arginine uptake by LSDM3 and ORS cells must be driven by something in addition to the membrane potential, since the cells accumulate arginine 10-fold relative to the extracellular concentration, and the membrane potential would be expected to cause only a 3-fold concentration. The additional driving force is not a Na^+ gradient, as demonstrated in this thesis, so it is likely that heteroexchange is occurring to drive arginine uptake. Alternatively, it is possible that the high values of arginine uptake observed might be due to arginine metabolism once it has been transported into the cells. To avoid this, perhaps the use of chemical analysis rather than radioactive measurement of the intracellular accumulation of arginine would have been a better method, but this was not feasible within the bounds of this project.

Since arginine is one of the key amino acids which is important for hair follicles, it is useful to know that system y^+ is responsible for its uptake in hair follicles, as it gives us some insight into its regulation and characteristics. The gene for the "housekeeping" form of y^+ , mCAT1, has previously been shown to be expressed in hair follicles and ORS cells (Chapter IV), whereas the other isoform, mCAT2, which is more restricted in tissue expression, has not been looked for in hair follicles. If mCAT2 is

present, it might be inducible, since mCAT2 is known to be inducible in tissues that also up-regulate nitric oxide synthase (*MacLeod et al., 1994*). However, it is possible to regulate mCAT1 by altering the membrane potential, so it may be feasible to increase the uptake of arginine by hair follicles whichever isoform of y^+ is involved.

The role of system y^+ in arginine transport suggested that the hair-growth-promoting drug minoxidil, which is believed to be an agonist of K^+ -channel activity (*Quast & Cook, 1989*), might affect arginine transport, as K^+ -channel opening is likely to alter the membrane potential, making it more negative. This hyperpolarisation would be expected to increase the driving force for arginine uptake. However, when the uptake of arginine was measured in the presence and absence of minoxidil at 10 or 100 $\mu\text{g/ml}$, no effect was seen. This level of minoxidil was tested because 10 $\mu\text{g/ml}$ minoxidil is effective at causing an increase in proliferation of 3T3 fibroblasts (*Sanders et al., in press*). However, the actual agonist is minoxidil-sulphate (*Buhl et al., 1990*), and there may not be enough time for sulphation to occur in these experiments. Therefore, it is possible that minoxidil does not cause a sufficient alteration in cell membrane potential to have any effect. Alternatively, the membrane potential may not actually be significantly altered by K^+ , as discussed above. It is important to clarify this, by using an indicator such as TPP^+ to determine whether the cell membrane potential does actually change at the different potassium concentrations, as discussed previously.

In LSDM3 but not ORS cells, histidine/leucine exchange was observed, which is characteristic of system y^+L , a recently identified transporter (*Devés et al., 1992*) (see table 1.1). This shows that LSDM3 cells are not identical in their transport properties to ORS cells, so care should be taken when extrapolating results from one cell-type to another. System y^+L can be distinguished from y^+ , because only y^+ is sensitive to *N*-

ethylmaleimide (NEM) (Devés *et al.*, 1993), so it would be possible to clarify that LSDM3 cells possess system y^+L by seeing if NEM abolishes arginine transport without affecting the histidine/leucine exchange.

Serine uptake was also rapid, at an initial rate of 280 pmol/well/min. The almost complete inhibition caused by excess cysteine confirms that serine and cysteine use the same transport system for cellular uptake, and this enables cysteine transport characteristics to be inferred to some extent by looking at serine transport, *i.e.* considering serine as an analogue of cysteine for the purposes of transport. The rapid and Na^+ -dependent transport of cysteine or serine observed is characteristic of system ASC. As with system y^+ , the gene for the ASC transporter was previously shown to be expressed in hair follicles and ORS cells (Chapter IV), so these results are consistent. To be certain that ASC is totally responsible for cysteine/serine transport, kinetic parameters such as K_m and V_{max} could be measured, but for the purposes of this thesis, it is sufficient to know that hair ORS cells will actively transport serine and cysteine, and that these amino acids compete for uptake.

The great variation in the measured rates of cysteine transport was surprising, as the behaviour of the cell-line appeared fairly constant, in terms of proliferation, morphology and transport of other amino acids. The cell-line is not expected to change significantly between cultures, unlike the variation that might be expected between different cultures of ORS cells derived from different subjects, so it is not likely that the variation in results is merely a reflection of variable cell behaviour. One possible reason for the variation could be that the cells vary in their requirements for cysteine throughout the cell cycle, but this seems unlikely to be the cause, as transport was measured at a similar time after cell seeding in each experiment. In addition, the effect

of passage number was examined to see if cysteine transport rate altered with successive passages, but the variation within a passage was as great as that observed between passages. This suggests that the cysteine itself is responsible for the variable results. Experiments using the same batch of ^{35}S -cysteine with erythrocytes, whose transport behaviour is well-documented (*Young et al., 1979*), suggested that the ^{35}S -cysteine was actually degraded into sulphite ion derivatives (*Ellory C., personal communication*). The batch of ^{35}S -cysteine was therefore replaced with a fresh one, but the results were still variable. This variation in cysteine transport remains unclear and requires further investigation.

Glutamate uptake by ORS cells was markedly slower than for the other amino acids studied, the initial transport rate being 4 pmol/nmol leu/min. It is possible that glutamate is entering the cells by facilitated diffusion, rather than by an active mechanism, as its uptake did not appear to be Na^+ -dependent, nor was it significantly inhibited by aspartate or cystine, which compete for the classical glutamate transport systems, X_{AG}^- and x_c^- , respectively (*Berteloot & Maenz, 1990*). However, there appeared to be some glutamate trapped in the dead space, which may have obscured the difference in uptake in the presence versus absence of Na^+ , aspartate or cysteine. The intracellular concentration of glutamate after 5 minutes is only about 100 μM , based on the intracellular volume estimate of 1 μl . Since 100 μM glutamate was supplied to the cells, this does not represent an accumulation of glutamate against its concentration gradient into the cells, suggesting that transport is via a diffusional process rather than an active mechanism. As a comparison, the intracellular concentration of arginine after 5 min is 984 μM , where arginine was also added at 100 μM , which represents a 10-fold concentration of arginine into cells against its concentration gradient. Similarly, serine

reaches a concentration of 1130 μM after 5 min, which is approximately a 50-fold concentration above the 20 μM serine originally added. Thus, although these concentration values are very approximate, due to difficulties in measuring cell volume, it is possible that hair follicle cells are not capable of actively transporting glutamate. Since glutamate is believed to be an important amino acid in hair biology (*Wiechers, personal communication*) this finding is very interesting, as it suggests that transport into cells may be limiting. It is also consistent with the fact that glutamate cannot completely substitute for glutamine to support follicle growth *in vitro*. The transport of glutamine should be measured, as it is possible that this is the route by which follicles obtain glutamate. Glutamine is a substrate for system N, which also transports histidine and asparagine, but it is not known whether system N is present in hair follicles.

MeAIB is a model substrate for the transporter System A (*Barker & Ellory, 1990*), which, unlike System ASC, can tolerate the bulky methyl group on the α -amino nitrogen of the molecule. System A transports amino acids in a Na^+ -dependent manner. However, the uptake of MeAIB by ORS cells appeared to be only partially dependent on sodium. MeAIB is also known to interact with the IMINO transport system (*Bertran et al., 1994*), but this is also Na^+ -dependent, so cannot account for the Na^+ -independent component of MeAIB transport observed here. Background trapped MeAIB may explain why Na^+ -dependence was not fully observed. It is possible that some diffusion of MeAIB is occurring, as the methyl group confers some lipophilicity upon the molecule. However, it is probable that at least some of the MeAIB uptake is via system A, because system A is almost ubiquitous (*Guidotti et al., 1978*), and also uptake is quite active, apparently accumulating MeAIB against its concentration gradient (intracellular concentration after 20 min is approx. 744 μM , which is 1.5-fold higher than the 500 μM

added). System A is highly regulated by cells (*Barker & Ellory, 1990*), so this is likely to offer a route for altering amino acid transport. The substrates of System A are neutral amino acids such as proline and glycine. The effect of these neutral amino acids on MeAIB transport by ORS cells should be measured, to ascertain whether or not system A is involved, as there should be competition for transport.

In conclusion, amino acid transport is a complex process in ORS cells, occurring by both Na⁺-dependent and Na⁺-independent routes, with different groups of amino acids competing for the same transporter. Glutamate transport is much slower than that of arginine, cysteine or serine, and may thus limit the utilisation of glutamate by hair follicles. It may be possible to regulate arginine transport via alterations in the cell membrane potential, while neutral amino acid transport which occurs by system A is likely to be subject to other regulatory routes such as hormonal.

CHAPTER VI

GENERAL DISCUSSION

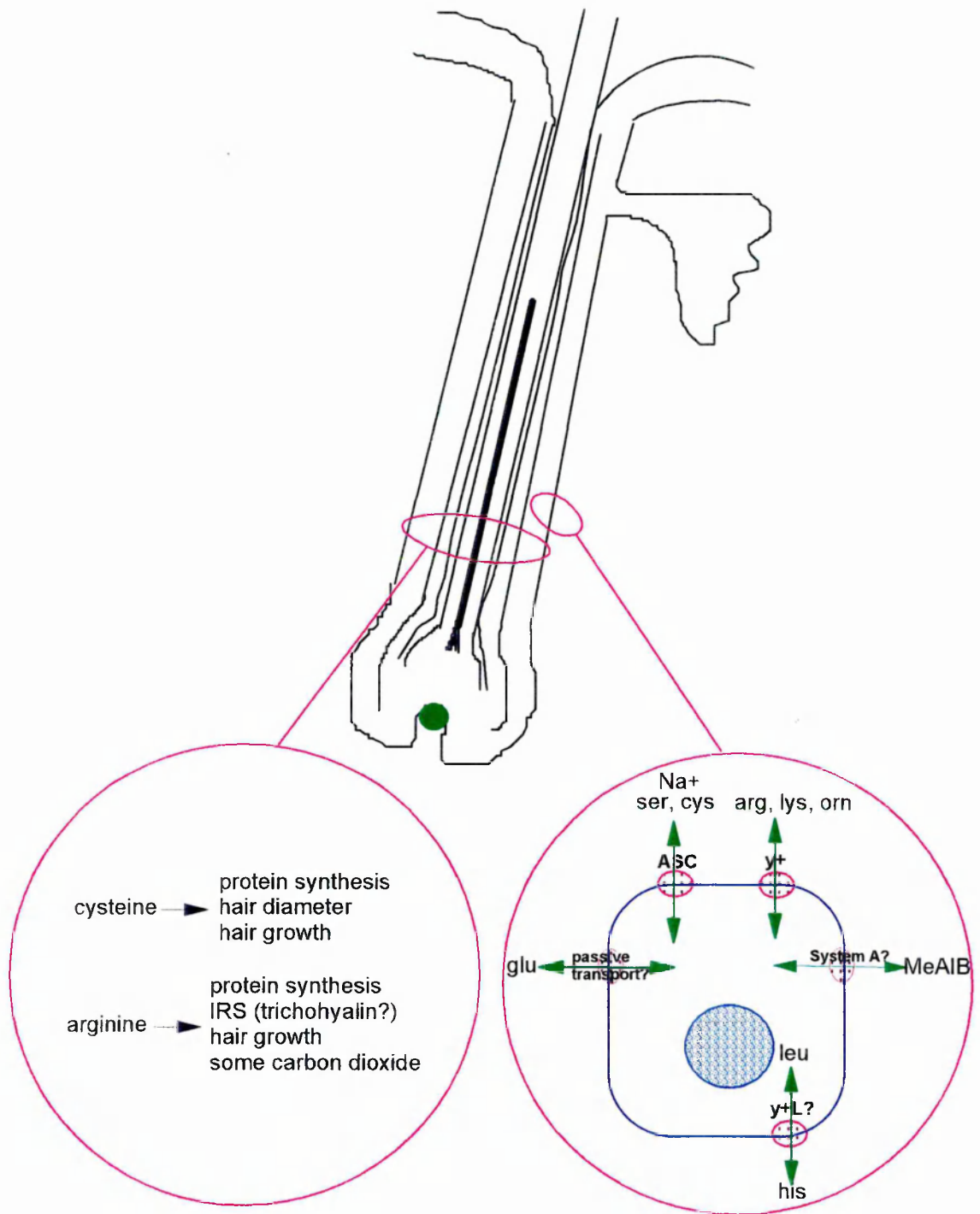
6.1 Introduction

The aim of this thesis was to investigate the role of amino acids in human hair follicles, in terms of their effects on hair growth and formation, their metabolism, their uptake and their subsequent localisation in follicles. The amino acid transport systems present in hair follicles were identified, so that the follicle uptake of amino acids could be understood at the cellular level. The amino acids cysteine and arginine were chosen as the main focus for the investigation, cysteine because it is known to be able to stimulate sheep wool production at the molecular level (*Fratini et al., 1994*), and arginine because it is a basic amino acid like glutamine, which is essential for hair follicle energy metabolism. In addition, glutamate was studied because it is a derivative of glutamine, and serine was investigated with respect to transport, as in other tissues it is transported by similar mechanisms to cysteine. The outer root sheath (ORS) was considered to be the first cell layer encountered by amino acids coming to the follicle from the blood, so ORS cells were cultured and used as a model system for measuring amino acid transport. Sections 6.2 to 6.4 summarise the major findings of this thesis, which are discussed in greater detail at the end of each relevant chapter, and figure 6.1 illustrates these findings diagrammatically. Finally, section 6.5 considers future work that could be carried out.

6.2 Role of Arginine in Human Hair Follicles

In chapter III it was demonstrated that arginine is essential for hair follicle

Figure 6.1: Summary Diagram



The figure depicts the main findings of this thesis. The importance of cysteine and arginine for hair follicles is shown, as are the mechanisms which have been identified for transport of amino acids into hair follicle outer root sheath cells. See text for details.

growth *in vitro*. Although hair follicles convert a small amount of arginine into carbon dioxide, the ultimate product of oxidative pathways, and in the absence of arginine follicles produce less lactate, the primary role of arginine does not appear to be metabolism for energy production. Rather, I suggest that the major role for arginine in the hair follicle seems to be a structural one. Arginine is actively taken up by hair follicles, and is subsequently localised primarily to the inner root sheath (IRS). It is therefore plausible that a significant role for arginine in the hair follicle is to form the arginine-rich protein, trichohyalin, a major component of the IRS.

6.3 Role of Cyst(e)ine in Human Hair Follicles

Chapter III also investigated the role of cysteine in hair follicles. The presence of cyst(e)ine was shown to be essential for hair growth and soluble protein synthesis *in vitro*. When the level of cysteine was reduced, not only was linear hair fibre growth retarded at low cysteine levels, but the fibre diameter also decreased. This effect was reversible, enabling a hair fibre to be grown with a thin region along its length corresponding to the part of the fibre formed at low cysteine. This suggests that cysteine supply regulates the diameter of the hair fibre being formed.

6.4 Amino Acid Transport in Hair Follicles and Related Cells

Chapter IV demonstrated that hair follicles, cultured outer root sheath cells and LSDM3 cells each express genes for the amino acid transporters ASC and y^+ (mCAT1), but suggested that the peptide transporters, PepT1 and HPT-1, and the putative transport subunit/activator encoded by rBAT are not expressed.

The presence of the actively-driven transport systems ASC for cysteine and y^+

for arginine is consistent with the belief that these amino acids are important to hair follicles. These transporters will enable follicles to actively scavenge these key amino acids from the blood supply, and accumulate them against their concentration gradients. Transport measurements in ORS cells in Chapter V were consistent with these molecular findings. Arginine transport by ORS cells was found to be rapid, Na^+ -independent, and inhibited by excess lysine or ornithine. These results are consistent with system y^+ transport, although the effects of cell membrane depolarisation were not clearly demonstrated. Since arginine is important for hair follicles, it is useful to know that system y^+ is involved in its uptake, as it gives us some insight into its regulation and characteristics. Similarly, the rapid and Na^+ -dependent serine transport observed, and its almost complete inhibition by excess cysteine, is characteristic of system ASC.

In addition, transport of glutamate was measured in ORS cells. Glutamate uptake was found to be markedly slower than for the other amino acids studied, and it is possible that it is entering the cells by facilitated diffusion, rather than by an active mechanism, as its uptake was not Na^+ -dependent, nor was it inhibited by aspartate or cystine, which compete for the classical glutamate transport systems, X_{AG} and x_c^- , respectively (Berteloot & Maenz, 1990). This implies that transport of glutamate into cells may be limiting for its metabolism by hair follicles, and it may explain why glutamate cannot substitute for glutamine to support follicle growth *in vitro* (Parmar P., personal communication).

To see if transport system A is present in hair follicles, uptake of the system A model-substrate, MeAIB, by ORS cells was measured. Although MeAIB transport was found to be only partially Na^+ -dependent, it is probable that at least some of the Na^+ -

dependent portion of the MeAIB uptake measured in ORS cells is via system A, because system A is almost ubiquitous (*Guidotti et al., 1978*). System A is subject to considerable regulation by cells, so this is likely to offer a route for altering neutral amino acid transport.

Thus, amino acid transport is a complex process in ORS cells, occurring by both Na⁺-dependent and Na⁺-independent routes, with different groups of amino acids competing for the same transporter. It may be possible to regulate the uptake of amino acids by hair follicles, for example by altering the cell membrane potential to affect arginine transport, or by altering the relative concentrations of competing amino acids such as serine and cysteine to manipulate cysteine uptake *in vitro*, or by supplying different levels of certain growth factors or hormones, such as IGF-1 or insulin, in particular to affect system A transport.

6.5 Conclusions and Future Work

This thesis opens up several interesting avenues for further investigation. The role of arginine in the IRS needs to be clarified. For example, it would be possible to separate the proteins of the IRS by polyacrylamide-gel-electrophoresis, after labelling follicles with ¹⁴C-arginine, to identify the proteins which are labelled. In addition, it would be interesting to see whether the metabolism of arginine to CO₂ can be regulated by altering the availability of other preferred substrates such as glutamine, as discussed in Chapter III.

The mechanism by which cysteine affects the hair fibre diameter needs to be elucidated. Its importance may be in the theory that, as follicles age, their blood supply, and thus their nutrient supply, becomes diminished. If this causes the cysteine supply

to become limiting, it may lead to thinning of hair fibres, and this may be responsible for the decline in hair thickness perceived with age (*Miyamoto et al., 1990*). Amino acid levels in blood increase with age (*Obled & Arnal, 1991*), implicating reduced transport into tissues. A decline in amino acid transport function with age has also been suggested, in other tissues such as gut (*Al-Mahroos et al., 1990*) and the blood-brain barrier (*Ito et al., 1995*). Perhaps the maintenance of hair fibre diameter depends on a satisfactory supply of cysteine to the follicle cells, and could be optimised by maximising the cellular uptake by system ASC. To be certain that ASC is totally responsible for cysteine/serine transport, kinetic parameters such as K_m and V_{max} could be calculated, by measuring the effect of concentration on transport. It may be that several transporters are involved in cysteine transport, and that regulation of these can determine hair fibre diameter.

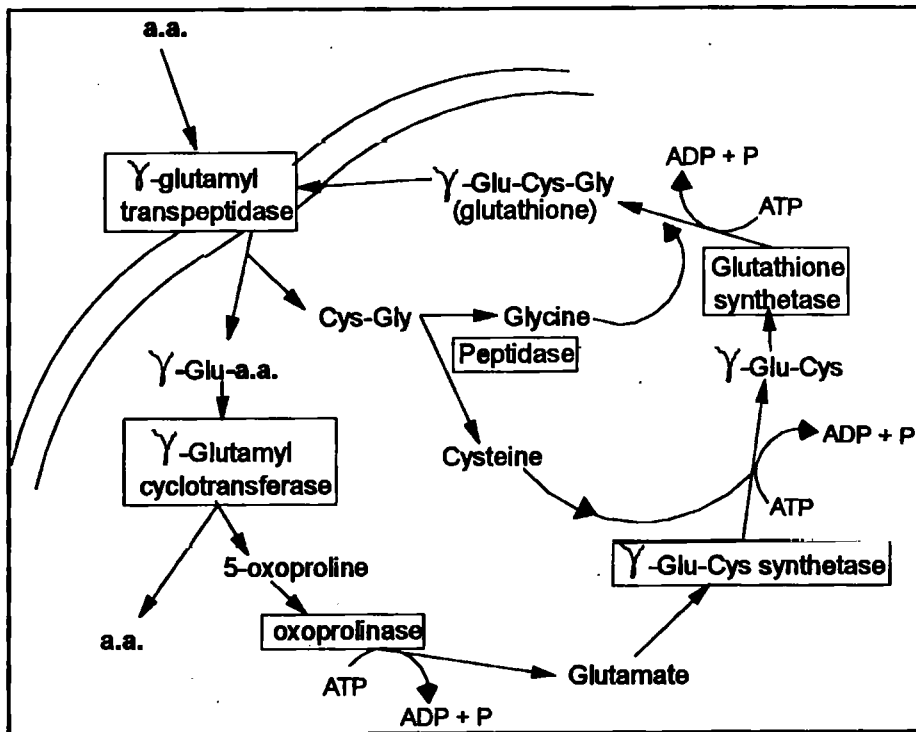
The effects of putative regulators of transport could be investigated, by measuring their effects on the time-course (pool accumulation & initial rate) of amino acid uptake. Minoxidil did not appear to affect transport of glutamate or arginine in preliminary experiments, although the form which is known to be active in stimulating hair growth, minoxidil-sulphate, should be tested (*Buhl et al., 1990*). Other possible regulators could include insulin, hydrocortisone, chloroquine (which has been shown to inhibit lysine uptake and enhance glutamate uptake by rat renal brush border membrane vesicles by *Chesney & Budreau, 1995*), or phytochemicals (*e.g.* certain herb extracts have been shown to promote hair regrowth in mice, *Inaoka et al., 1994*, and it is possible that they could act via stimulation of transport), as well as other competitive amino acids and changes in pH or membrane potential.

Recent evidence showing cysteine incorporation into follicles primarily at the

keratinizing region (*Philpott M., personal communication*) suggests that expression of system ASC (or other cysteine transporters) may vary along the length of the hair follicle, perhaps being specifically expressed in the keratinizing zone. *In situ* PCR, or PCR on follicle pieces from different regions of the follicle, would help to clarify this "zonal uptake". Transport of labelled cysteine or serine into follicle pieces from different regions of the follicle could be attempted, to compare the rate of uptake in different regions.

Another possible route for follicles to obtain sulphur is by the uptake of cystine. The transport system responsible for cystine uptake is system x_c^- , which is competitively inhibited by glutamate. This could be a problem if we wish to provide both cystine and glutamate to hair follicles. However, the finding that glutamate uptake is not Na^+ -dependent, and that is not inhibited by excess cystine, suggests that system x_c^- is not present in hair follicles (see section 6.4). One possibility is that the γ -glutamyl cycle is responsible for cystine transport in hair follicles. This cycle (figure 6.2) was proposed by Meister and colleagues (*Orlowski & Meister, 1970; Meister, 1973*), as an additional role for glutathione, the tripeptide γ -glutamylcysteinylglycine, which is found in nearly all cells. Glutathione is thought to be important for maintaining the sulphydryl groups of intracellular proteins in a reduced state. Its involvement in the γ -glutamyl cycle is thought to be of particular importance for the uptake of cysteine and methionine into cells. However, it is not universally accepted that the cycle is involved in amino acid transport. In a recent paper (*Sweiry et al., 1995*), evidence was obtained, for the first time, for γ -glutamyl transpeptidase mediated transport of cystine into a human cell line, supporting the proposed cycle. *Vina et al. (1989)* suggested that the γ -glutamyl cycle, in addition to being a mechanism for amino acid transport, may also generate a

Figure 6.2: The proposed γ -glutamyl cycle



The transport of the amino acid is proposed to involve a transpeptidation which transfers the glutamyl residue of glutathione onto the amino acid, converting the amino acid into a dipeptide. Glutamate is eventually reformed and combines with cysteine and then glycine to reform glutathione.

signal that subsequently activates amino acid transport proteins. One signal proposed was 5-oxoproline (see figure 6.2). In the Sweiry paper 5-oxoproline was found to cause a small but significant increase in cystine and glutamine transport into the cell line. Therefore, it would be interesting to investigate the involvement of the γ -glutamyl cycle in cystine transport in hair follicles, and to see whether 5-oxoproline is effective in stimulating the transport of other amino acids.

The molecular and functional studies presented in this thesis provide good evidence that system ASC is involved in the transport of cysteine, but the involvement of system A in the transport of MeAIB is not so clear. To ascertain whether or not system A is involved, experiments should be carried out to see whether or not neutral amino acids such as glycine compete with MeAIB for transport by ORS cells. In addition, the extent to which system A is responsible for transport of a given amino acid may be determined by measuring its uptake in the presence of excess MeAIB to block system A.

The transport of glutamine should be measured, as it is possible that this is the route by which follicles obtain glutamate. Glutamine is a substrate for system N, which also transports histidine and asparagine, but it is not known whether system N is present in hair follicles. Transport of histidine would also be of interest, since it is believed to be co-transported with zinc. Zinc supplementation has been shown to improve hair growth and strength in patients with alopecia (*Slonim et al., 1992*), although there are also reports of zinc having no effect on hair growth (*Medeiros et al., 1987*). It would therefore be interesting to investigate the co-transport of histidine and zinc, alongside a study of the effects of zinc on *in vitro* hair follicle growth and metabolism.

Further molecular biology of transporters could be carried out as new ones are

cloned. For example, it is possible that follicles do express peptide transporters other than PepT1 and HPT-1, so the PepT2 gene should be probed for by PCR, and functional transport of radiolabelled peptides measured, before it can be stated conclusively that peptides are not substrates for follicle transport mechanisms. The other isoform of system y^+ , mCAT2, which is more restricted in tissue expression, could also be looked for. If mCAT2 is present, it might be inducible, since mCAT2 is known to be inducible in tissues that also induce nitric oxide synthase, which uses arginine as a substrate (MacLeod *et al.*, 1994). In addition, *in situ* work or quantitative PCR could be performed to give information about the location or relative expression levels of transporters.

In conclusion, the findings of this thesis open the way to explaining why certain amino acids are important for supporting hair growth and formation, and how these amino acids are taken up by the cells of the hair follicle, and will facilitate the identification of the conditions necessary for optimal hair growth.

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